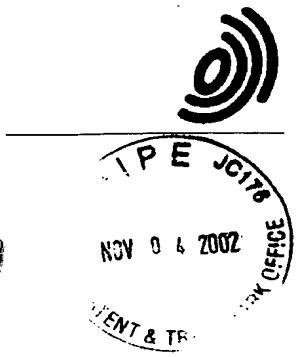

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

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Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 d r Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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3

NEW IMMUNOPROTECTIVE INFLUENZA ANTIGEN AND ITS USE IN VACCINATION

The present invention relates to new immunoprotective influenza antigens, which are non-existent in nature. The invention further relates to the use of the antigens for vaccination and to vaccines containing them, as well as to methods for preparing the antigens.

Influenza is caused by an RNA virus of the myxovirus group. Influenza viruses can be classified into three types (A, B and C), based on antigenic differences in the nucleoprotein and the matrix protein. Type A and B influenza viruses each contain 8 RNA segments, while type C only has 7 RNA segments. Influenza A is most important and is very pathogenic for man, as well as for animals, for example pigs and horses. Type B influenza causes disease in humans. Influenza C is less severe and was isolated from humans and pigs. The virus is transmitted through the air, mainly in droplets expelled during coughing and sneezing. The influenza viruses cause an infection of the respiratory tract, that is usually accompanied with coughing, high fever and myalgia. Although an influenza infection does not often lead to the death of the infected individual, the morbidity can be severe. As a consequence thereof influenza epidemics may lead to substantial economic loss. Furthermore, influenza infection can be more dangerous for certain groups of individuals, such as those having suffered from a heart attack, CARA patients or elderly. A vaccine against influenza is therefore highly desirable.

The influenza A virus contains in its membrane two highly immunogenic, but very variable proteins, the haemagglutinin and the neuraminidase. Due to the variability of these two proteins a broad spectrum long lasting vaccine against influenza A has so far not been developed. The influenza vaccine commonly used, has to be adapted almost every year to follow the antigenic drift

of the virus. In these circumstances the vaccine can protect about 80% of the immunised persons. When more drastic changes occur in the virus, known as antigenic shift, the vaccine is no longer protective.

It is therefore the object of the present invention to provide a new immunoprotective antigen for use in vaccines which is not based on the rapidly changing haemagglutinin and/or neuraminidase and which therefore lacks the disadvantages of these known antigens and vaccines based thereon.

In the research that led to the present invention it was found that well conserved membrane proteins of influenza other than haemagglutinin and neuraminidase can be used for eliciting protection. Particularly useful for this approach is the membrane protein M2.

M2 mRNA is encoded by RNA segment 7 of the influenza A virus. It is encoded by a spliced mRNA (Lamb et al., 1981). Like the haemagglutinin and the neuraminidase, the M2 protein is an integral membrane protein of the influenza A virus. But the protein is much smaller, only 97 amino acids long. 24 amino acids at the amino terminus are exposed outside the membrane surface, 19 amino acids span the lipid bilayer, while the remaining 54 residues are located on the cytoplasmic side of the membrane (Lamb et al., 1985).

The M2 protein is abundantly expressed at the cell surface of influenza A infected cells (Lamb et al., 1985). The protein is also found in the membrane of the virus particle itself, but in much smaller quantities, 14 to 68 molecules of M2 per virion (Zebedee and Lamb, 1988). The M2 protein is posttranslationally modified by the addition of a palmitic acid on cysteine at position 50 (Sugrue et al., 1990).

The M2 protein is a homotetramer composed of two disulfide-linked dimers, which are held together by noncovalent interactions (Sugrue and Hay, 1991). By site-directed mutagenesis, Holsinger and Lamb (1991)

demonstrated that the cysteine residue at position 17 and 19 are involved in disulfide bridge formation. Only cysteine at position 17 is present in all viruses analysed, therefore it seems likely that this is the most important residue. In the virus strains where cysteine 19 is also present, it is not known whether a second disulfide bridge is formed in the same dimer (linked by Cys 17 - Cys 17) or with the other.

By aligning the sequences of M2 proteins, isolated from different human strains of influenza A virus, a striking conservation of the extracellular part of the M2 protein, became evident (table 1). Since the first human influenza A strain isolated in 1933, A/WS/33 (H1N1), until the most recently sequenced virus A/Guangdong/39/89 (H3N2), no amino acid change has been observed in the extracellular domain of the M2 protein. Two virus strains do not fit in this conserved pattern, A/PR/8/34 (H1N1), which shows one amino acid change and A/Fort Monmouth/1/47 (H1N1), which shows three amino acid differences. These two strains probably represent side branches in the evolutionary tree.

Table 1 gives an overview of the amino acid sequences of the extracellular domain of the influenza A M2 protein of the virus strains A/WSN/33 (Markushin et al. (1988)), A/PR/8/34 (Allen et al. (1980), Winter and Fields (1980)), A/WS/33, A/Fort Warren/1/50, A/Singapore/1/57 and A/Port Chalmers/1/73 (all described by Zebedee and Lamb (1989)), A/Udorn/72 (Lamb and Lai (1981)), A/Leningrad/134/57 (Klimov et al. (1992)), A/Ann Arbor/6/60 (Cox et al. (1988)), A/Bangkok/1/79 (Ortin et al. (1983)), A/New York/83 (Belshe et al. (1988)), A/Fort Monmouth/1/47 (EMBL U02084), A/USSR/90/77 (EMBL X53029) and A/Guangdong/39/89 (EMBL L 18999).

Table 1

Amino acid sequence of the extracellular domain of the M2 protein

Strain	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A/WS/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/WSN/33 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/PR/8/34 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Gly	Ser	Ser	Asp
A/Fort Monmouth/1/47 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Thr	Lys	Asn	Glu	Trp	Glu	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Fort Warren/1/50 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/USSR/90/77 (H1N1)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Singapore/1/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Leningrad/134/57 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Ann Arbor/6/60 (H2N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Udorn/72 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Port Chalmers/1/73 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Bangkok/1/79 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/NY/83 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp
A/Guangdong/39/89 (H3N2)	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile	Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Asp	Ser	Ser	Asp

It was anticipated by the present inventors that the conserved character of this type of membrane proteins could make them a good candidate for vaccine development. In principle the protective capacity of anti-M2 antibodies is already known. Experimental data demonstrated that a monoclonal antibody directed against the extracellular part of the M2 protein (14C2) can diminish the spread, although the infectivity of the virus in vitro was not reduced (Zebedee and Lamb, 1988). Furthermore it was demonstrated that passively administered monoclonal antibody (14C2) could inhibit viral replication in the lungs of mice (Treanor et al., 1990). Both approaches rely on the administration of anti-M2 antibodies. However, the passive administration of monoclonal antibodies is preferably avoided because of the immunogenicity of heterologous immunoglobulins which, upon repeated administration, can lead to the clearing of the antibodies from the body and thus to a reduction of the efficacy of the treatment. Even homologous antibodies can elicit anti-idiotype antibodies. Furthermore, it was found that humans infected with the virus do have anti-M2 antibodies but these do not protect against infection, (either their concentration or their nature are not sufficient to confer efficacy). This makes it unlikely that passive administration of anti-M2 antibodies is suitable for use in humans. It also teaches away from trying to develop vaccines for humans based on this antigen.

Recently, protection of mice against an infection with homologous or heterologous virus was described (Slepushkin et al., 1995). These authors used a formulation of incomplete Freund's adjuvant and a membrane extract of Sf9 cells expressing the complete M2 protein for immunisations. However, this approach is also not suitable for vaccination of humans because it relies on the use of Freund's adjuvant which is prohibited in humans.

In summary, use of antibodies for providing protection against influenza is preferably to be avoided. Moreover, it is unlikely that prophylactic treatment with antibodies will be effective in humans. Immunisation with complete M2 protein in humans as described is not possible because it relies on incomplete Freund's adjuvant which cannot be used in humans, and is counter-indicated in higher animals.

It is thus the object of the invention to provide for an alternative influenza antigen that is sufficiently immunoprotective and is not dependent on Freund's adjuvant to find a use in humans.

According to the invention it has now been found that it is possible to prepare such a novel antigen that does not exist in nature. For this the extracellular part of a conserved influenza membrane protein or a functional fragment thereof is fused to a presenting carrier, for example a (poly)peptide. The conserved influenza membrane protein is for example the well conserved, extracellular part of the M2 protein. The membrane protein is preferably genetically fused to a presenting (poly)peptide as the presenting carrier, which (poly)peptide stabilises the extracellular part and surprisingly potentiates the immunogenicity of the fusion product thus obtained. It is thought that the presenting (poly)peptide brings the extracellular part into its wild type structure, thus presenting the antigen in a form that is also found on the virus and on the infected cells.

A 'functional fragment of the conserved influenza membrane protein' is a fragment that is capable of eliciting a statistically significant higher immunoprotection when administered in an immunoprotective dose to test members of a species than is found in control members of the same species not receiving the functional fragment.

In one embodiment of the invention the 23 amino acid extracellular part of the M2 protein is fused to the

amino terminus of the human Hepatitis B virus core protein. In this way the wild type structure of the M2 protein in viral particles and on infected cells is mimicked, where the free N-terminus extends in the extracellular environment.

Alternative presenting (poly)peptides are multiple C3d domains (Dempsey et al., 1996), tetanus toxin fragment C or yeast Ty particles. 'Presenting (poly)peptides' are intended to encompass every stretch of amino acid(s) that can present the extracellular part, in a substantially wild type form, towards the environment.

Alternatively, the presenting carrier can be a non-peptidic structure, such as glycans, peptide mimetics, synthetic polymers, etc..

After expression of the novel antigen in a suitable acceptor cell, it can be used either as such (depending on the acceptor cell), as part of a membrane fragment or in isolated form.

The term 'presenting carrier' is used to indicate all types of presenting molecule, both (poly)peptides and others.

It will be clear for the person skilled in the art that a gene construct, comprising the coding information for the antigen and the presenting (poly)peptide, can not only be used to prepare the new antigen, as described above, but that it can also be used, optionally in the presence of suitable transcription and/or translation regulatory sequences, in a DNA vaccine, or in vaccinia based vaccine constructions.

A presenting (poly)peptide can be incorporated into the fusion product in a single copy or in multiple copies. The third complement protein fragment d (C3d) is preferably used in more copies, preferably 3.

In a preferred embodiment of the invention the fusion product further may comprise an additional peptide at an appropriate internal site (Schödel et al., 1992) or

C-terminal (Borisova et al., 1989). This additional peptide is intended to further increase the protective capacity of the antigen, and may for example be a T helper cell epitope or a cytotoxic T cell epitope.

The antigen of the invention is obtainable by preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and optionally the coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation and/or secretion regulatory sequences, bringing this gene construct in a suitable acceptor cell, effecting expression of the gene construct in the acceptor cell and optionally isolating the antigen from the acceptor cell or its culture medium.

The presence of the transcription and/or translation and/or secretion regulatory sequences depends on whether the gene is to be integrated into a vector or the genome of the acceptor cell at a position already providing these signals.

The coding sequence for a presenting (poly)peptide is only present when the fusion product is a fusion between the antigen and a peptidic structure and if it is desirable to directly link the two structures in the DNA construct. In all other instances, the presenting carrier may be added to the antigen in a different manner.

The suitable acceptor cell can be selected from E. coli, Lactococcus lactis, Lactobacillus plantarum, yeast (e.g. Pichia pastoris). In the case of L. lactis the antigen need not be isolated but the engineered bacteria can be used directly for intranasal or oral use.

The invention further relates to vaccines that comprise at least the antigen of the invention. This antigen can be in isolated form or being part of a membrane fragment or being expressed on the acceptor cell. The antigen of the invention can be used together

with suitable excipients. The person skilled in the art of vaccine design will be capable of selecting suitable excipients. Guidance may for example be found in Methods in molecular medicine: Vaccine Protocols (1996). Eds. Robinson, A., Farrar, G.H. and Wiblin, C.N. Humana Press, Totowa, New Jersey, USA.

The antigens of the invention may be used alone or in combination with one or more other influenza antigens, such as neuraminidase, haemagglutinin or native M2.

Furthermore, the invention relates to the use of the antigens in the preparation of a vaccine against influenza. The vaccines can be direct vaccines, i.e. vaccines containing the fusion products or indirect DNA vaccines. The latter are vaccines, comprising the fusion cDNA under the regulation of a eukaryotic promoter that can function in the recipient. The actual antigen is then produced in the recipient of the vaccine.

The vaccines of the invention are intended both for use in humans and in animals, for example pigs and horses of which it is known that they are infected by influenza A.

A similar approach as described here for preparing novel fusion antigens of influenza A can be adopted to prepare similar fusion antigens and vaccines containing the fusion antigens or DNA encoding the fusion antigens for influenza B and C.

The invention also relates to a method of preparing the antigens, comprising the steps of:

a) preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation and/or secretion regulatory sequences,

b) bringing this gene construct in a suitable acceptor cell,

c) effecting expression of the gene construct in the acceptor cell, and

d) optionally isolating the antigen from the acceptor cell or its culture medium.

The invention will be further illustrated by the following example, that is in no way intended to limit the invention. The example extensively describes the preparation of fusion proteins of M2 with various presenting (poly)peptides and the use thereof in immunisation. Instead of M2 and the presenting carriers described here, the skilled person will be capable of choosing another conserved influenza membrane protein and other presenting carriers.

In the example reference is made to the following figures:

Figure 1 : Construction of pATIPM2m1.
E1 and E2 = first and second exon of the influenza M2 protein,
M2e = extracellular part of the M2 protein,
M2t = transmembrane part; and
M2c = cytoplasmic tail.
Bold line = vector.
(a) removal of the intron out of the m2 gene,
(b) introduction of a Bcl I site between the extracellular part and the transmembrane domain of the M2 protein,
(c) nucleotide and amino acid sequence of the extracellular part of the M2 protein of A/PR/8/34.

Figure 2 : Construction of pIPM2hB2Mm2s2.
ori = origin of replication,
cat = chloramphenicol acetyltransferase,
bla = ß-lactamase,
lpp = lipoprotein,
hB2M = human $ß_2$-microglobulin,
ompa-ss = signal sequence of the outer membrane protein A,
ssDNA = single-stranded DNA,
M2e = extracellular part of the M2 protein.

(a) : Construction flow scheme,
(b) : Details of key sequences.

Figure 3 : Construction of pPLcIPM2HBcm.
ori = origin of replication,
cat = chloramphenicol acetyltransferase,
bla = ß-lactamase,
HBc = hepatitis B core,
ssDNA = single-stranded DNA,
M2e = extracellular part of the M2 protein.
(a) : Plasmid construction flow scheme,
(b) : Sequence around the introduced BamHI restriction site in the hepatitis B core gene,
(c) : Details of key sequences.

Figure 4 : Analysis of the soluble fraction, corresponding to 150 µl original culture, of strain MC1061[pcI857] containing the plasmids pPLc245 (control), pPLcA1 (expression of HBc) or pPLcIPM2HBcm (expression of IPM2HBcm) respectively, on a SDS 12.5% PAGE. After the electrophoresis the gel was stained with Coomassie brilliant blue.
MW = molecular weight marker,
NI = not induced culture,
I = induced culture.

Figure 5 : Analysis of the soluble fraction, corresponding to 150 µl original culture, of strain MC1061[pcI857] transformed with pPLc245 (control), pPLcA1 (expression of HBc) or pPLcIPM2HBcm (expression of IPM2HBcm) respectively, as in figure 4. After electrophoresis, the relevant proteins were revealed by a Western blotting experiment. Detection with (A) a monoclonal antibody against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein.
MW = molecular weight marker,
NI = not induced culture,
I = induced culture.

Figure 6 : Sequence of the amino terminus of the M2 protein compared to the amino terminus of

IPM2HBcm, as experimentally determined. Sequence of A/Udorn/72 (Lamb and Zebedee, 1985).

Figur 7 : Soluble fractions of strain MC1061[pcl857] transformed with pPLc245 (control), pPLcA 1 (expression of HBc) or pPLcIPM2HBcm (expression of IPM2HBcm), respectively, analysed in a native state by means of a dot blot. Detection with (A) a monoclonal antibody against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein.
NI = not induced culture,
I = induced culture.

Figure 8 : Overview of (A1) rectal temperature, (A2) weight and (B) survival of the mice vaccinated with IPM2HBcm after a lethal challenge with 5 LD_{50} m.a. A/PR/8/34. The statistical significance was calculated by the Fisher's exact test. Mice immunised with different doses of antigen were compared to the control group. The following results were obtained: for 50 μg IPM2HBcm $p<0.001$; for 10 μg $p<0.005$ and for the 5 μg dose $p<0.05$. Figure 8C shows the survival of the mice vaccinated intraperitoneally with IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with 30 HAU X-47. Figure 8D shows the survival of the mice vaccinated intranasally with IPM2HBcm, and IM2HBcm, respectively, after a lethal challenge with 30 HAU X-47.

Figure 9 : Analysis of the serum samples of the four set ups reported in figure 8. The pre-immune serum (a), the serum taken after the first (b), after the second (c) and after the third (d) immunisation and the serum taken after challenge (e) were initially diluted 1/50. The consecutive dilution steps were 1/3. The plotted absorbance is a corrected value obtained as described in Results, Analysis of the serum samples.

Figure 10 : Construction of pPLcIM2HBcm.
ori = origin of replication,
cat = chloramphenicol acetyltransferase,
bla = ß-lactamase,
M2e = extracellular part of the M2 protein,

HBc = hepatitis B core.

Figure 11 : Analysis of the soluble fraction, containing 5 μg HBc or I(P)M2HBcm (as determined in an ELISA (see Materials and methods)), of strain MC1061 [pcI857] containing respectively the plasmids pPLc245 (control), pPLcA1 (expression of HBc), pPLcIPM2HBcm (expression of the fusion protein IPM2HBcm with the extracellular part of the M2 protein derived from A/PR/8/34) or pPLcIM2HBcm (expression of IM2HBcm, containing the more universal M2 sequence) on a SDS 12.5% PAGE-gel.
MW = molecular weight marker,
NI = not induced,
I = induced culture.

Figure 12 : Analysis of the soluble fraction, containing 2.5 μg HBc or I(P)M2HBcm (as determined in an ELISA (see Materials and methods)), of strain MC1061 [pcI857] containing respectively the plasmids pPLc245 (control), pPLcA1 (expression of HBc), pPLcIPM2HBcm (expression of the IPM2HBcm) or pPLcIM2HBcm (expression of IM2HBcm) on a Western blot (see Materials and methods). Detection with (A) a monoclonal antibody directed against HBc and (B) a monoclonal antibody specific for the extracellular part of the M2 protein.
MW = molecular weight marker,
NI = not induced,
I = induced culture.

Figure 13 : Overview of the oligonucleotides used for PCR amplification of hbc and i(p)m2hbc. 's' or 'a' following the name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed sequence indicates the changed Leu codons.

Figure 14 : Overview of the construction of hbc and m2hbc fusions in vectors for L. lactis.
ori = origin of replication for E. coli,
ori(+) = origin of replication for L. lactis,
ermA and ermM = erythromycin resistance genes,

P1 = L. lactis promoter,
bla = ß-lactamase,
HBc = hepatitis B core,
M2e = extracellular part of the M2 protein,
usp45-ss = signal sequence of usp45,
mIL2 = murine interleukin 2 and
mIL6 = murine interleukin 6.

Figure 15 : Analysis of the expression of Hepatitis B core (HBc) and M2-HBc fusion proteins in a Western blot. An equivalent of 10^9 L. lactis bacteria of strain MG1363 containing respectively pTREX1 (control), pT1HBc, pT1HBcIL2, pT1HBcIL6 (expression of HBc alone or in combination with mIL2 or mIL6, respectively), pT1PM2HBc, pT1PM2HBcIL2, pT1PM2HBcIL6 (expression of IPM2HBcm alone or in combination with mIL2 or mIL6, respectively), pT1M2HBc, pT1M2HBcIL2, pT1M2HBcIL6 (expression of IM2HBcm alone or in combination with mIL2 or mIL6, respectively), was analysed in a SDS 12.5% PAGE-gel. The first antibody, p-anti-HBc (Dako Corporation, Carpinteria, CA., USA) was diluted 5000 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-rabbit IgG labeled with alkaline phosphatase (Southern Biotechnology Associates, Birmingham, AL., USA). I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.
MW = molecular weight marker,
C = control and
- = expression of the antigen alone.

Figure 16 : Analysis of the expression of M2-HBc fusion proteins in a Western blot. An equivalent of 2 to $3x10^9$ L. lactis bacteria of strain MG1363 containing respectively pT1HBc (control), pT1PM2HBc, pT1PM2LHBc (expression of IPM2HBcm), pT1M2HBc, pT1M2LHBc (expression of IM2HBcm), was separated on a SDS 12.5% PAGE-gel. The fusion proteins were detected with an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and methods). The bound antibodies were detected with a 1/2000 dilution of the alkaline phosphatase conjugated

polyclonal anti-mouse IgG (γ-chain specific) (Southern Biotechnology Associates, Birmingham, AL., USA).
MW= molecular weight marker,
C = control,
E = leucine codons optimal for use in E. coli, and
L = leucine codons optimal for use in L. lactis.
These are the plasmids pT1PM2LHBc and pT1M2LHBc, respectively. I(P)M2HBc stands for either IPM2HBcm or IM2HBcm.

Figure 17 : Overview of the oligonucleotides used for PCR amplification of the extracellular part of the M2 protein and C3d.
's' or 'a' following the code name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

Figure 18 : Overview of the construction of m2c3d3 fusions in L. lactis.
ori = origin of replication for E. coli,
ori(+) = origin of replication for L. lactis,
ermA and ermM = erythromycin resistance genes,
P1 = L. lactis promoter,
bla = ß-lactamase,
M2e = extracellular part of the M2 protein,
usp45-ss = signal sequence of usp45,
spaX = anchor sequence derived from Staphylococcus aureus protein A,
C3d = complement protein 3 fragment d, and
mIL6 = murine interleukin 6.

Figure 19 : Overview of the oligonucleotides used for PCR amplification of ttfc and m2ttfc.
's' or 'a' following the name of the oligonucleotide stands for the use of these primers in the sense (s) or anti-sense (a) orientation. The boxed region indicates the changed Leu codons.

Figure 20 : Overview of the construction of m2ttfc in vectors for L. lactis.
ori = origin of replication for E. coli,

ori(+) = origin of replication for L. lactis,
ermM and ermμ = erythromycin resistance genes,
P1 = L. lactis promoter,
bla = ß-lactamase,
TTFC = tetanus toxin fragment C,
M2e = extracellular part of the M2 protein,
usp45-ss = signal sequence of usp45,
mIL2 = murine interleukin 2, and
mIL6 = murine interleukin 6.

Figure 21 : Analysis of the expression of IPM2TTFC fusion protein in a Western blot. An equivalent of 10^9 L. lactis bacteria of strain MG1363 containing respectively pT1TT (control), pT1PM2LTT (expression of IPM2TT), pT1PM2LTTIL2 (expression of IPM2TT in combination with mIL2) or pT1PM2LTTIL6 (expression of IPM2TT in combination with mIL6), was analysed in a SDS 10% PAGE-gel. The first antibody, an IgG fraction of a polyclonal mouse anti-M2e antibody (see Materials and methods) was diluted 2500 times. The bound antibodies were detected with a 1/2000 dilution of the polyclonal anti-mouse IgG labeled with horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL., USA). 30 mg 4-chloro-1-naphthol (Sigma Chemical Co., St. Louis, Mo., USA), was solubilised in 10 ml methanol. Afterward 40 ml PBS, pH 7.4 and 150 μl H_2O_2 was added.
MW = molecular weight marker,
- = expression of the antigen alone,
mIL2 = expression of the antigen in combination with mIL2,
mIL6 = expression of the antigen in combination with mIL6.

The following abbreviations will be used:

1 LD_{50}	:	the viral challenge required to kill half of the population of infected mice
BCIP	:	5-bromo-4-chloro-3-indolylphosphate
bp	:	base pair(s)
CIP	:	calf intestine phosphatase
C3d	:	complement protein 3 fragment d

DEA	:	diethylamine
HAU	:	haemagglutination units
hB2M	:	human ß2-microglobulin
HBc	:	Hepatitis B core protein
IM2HBcm	:	universal influenza A M2 protein fragment fused to HBc
IPM2hB2Mm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to hB2M
IPM2HBc	:	influenza A M2 protein fragment (from A/PR/8/34), fused to HBc, containing four additional amino acids between the first methionine and the start of the extracellular part of the M2 protein
IPM2HBcm	:	influenza A M2 protein fragment (from A/PR/8/34) fused to HBc
IPTG	:	isopropyl-ß-D-thiogalactoside
m.a.	:	mouse adapted
M2C3d3	:	universal influenza M2 fragment fused to three copies of C3d
cM2C3d3	:	cytoplasmic form of M2C3d3
sM2C3d3	:	secreted form of M2C3d3
sM2C3d3X	:	form of M2C3d3 covalently attached in the cell wall
MES	:	2-(N-morpholino)ethanesulphonic acid
MPLA	:	monophosphoryl lipid A
NBT	:	nitro blue tetrazolium
OmpA-ss	:	signal sequence of the outer membrane protein A
PCR	:	polymerase chain reaction
SDS-PAGE	:	sodium dodecylsulfate polyacrylamide gel electrophoresis
TDM	:	trehalose dicorynomycolate

EXAMPLE

INTRODUCTION

This example demonstrates the preparation of various fusion antigens based on the influenza A virus M2 protein. The M2 fragment was fused to the amino terminus of various presenting carriers.

MATERIALS AND METHODS

1. Bacterial strains and plasmids

All plasmid constructions, made for expression in Escherichia coli, were performed in strain MC 1061 (hsdR mcrB araD139Δ(araABC-leu)7697 ΔlacX74 galU galK rpsL thi (Casadaban and Cohen, 1980) because of high efficiency of transformation. The first transformation after mutagenesis was performed in WK6λmutS (Δ(lac-proAB), galE, strA, mutS::Tn10/lacI^q, ZΔM15, proA$^+$B$^+$; Zell and Fritz, 1987). Expression studies of human $ß_2$-microglobulin and derivatives were performed in E. coli strain C3000 (Hfr, sup$^-$, thi(λ$^-$)). Expression studies of the Hepatitis B core protein and derivatives were carried out in MC1061 [pcI857].

pcI857 was described in Remaut et al., 1983b. A derivative of this plasmid pcI857K1 was described in Steidler et al., 1994.

The plasmid p714 (Parker and Wiley, 1989) was a kind gift of Dr. K. Parker and the plasmid pPLcA1 (Nassal, 1988) of Dr. M. Nassal. The plasmid pPLc245 was described in Remaut et al., 1983a.

For the constructions and expressions in Lactococcus lactis strain MG1363 (Gasson, 1983) was used. The vector for constitutive expression in L. lactis, pTREX1 (Wells and Schofield, 1996) was a generous gift from Dr. K. Schofield. The plasmid pL2MIL2, for the expression of interleukin 2, is described in Steidler et al., 1995. An analogous plasmid for the expression of interleukin 6, pL2MIL6, is described in Steidler et al., 1996.

The vector pSG5.C3d.YL (Dempsey et al., 1996) is a gift from Dr. Fearon.

2. Virus

A/PR/8/34 (H1N1) was adapted to mice by several lung passages. After adaptation, the virus was grown in eggs (Kendal et al, 1982) and purified over a sucrose gradient. The titer (haemagglutination units (HAU) (Hirst, 1941; Kendal et al, 1982) and the lethality in mice were determined. For m. a. A/PR/8/34, 1 LD_{50} corresponded to 10 HAU present in 50 μl.

Influenza strain X-47 (H3N2) (Baez et al., 1980) was used in experiments for heterologous challenge. This strain was adapted to mice by several lung passages.

3. Animals

Female Balb/c mice were purchased from Charles River Wiga (Sulzfeld, Germany). The mice were used at the age of 6 to 7 weeks.

4. Antibodies

The monoclonal mouse antibody directed to the Hepatitis B core protein was a kind gift from Dr. Sc. H. Claeys (Bloedtransfusiecentrum, Leuven).

A mouse monoclonal antibody specific for the human $ß_2$-microglobulin was purchased from Boehringer (Mannheim, Germany).

Alkaline phosphatase conjugated antibodies specific for mouse IgG or mouse IgG (γ chain specific) were bought from Sigma Chemical Co. (St. Louis, Mo., USA).

5. Growth media

E. coli was grown in LB medium (1% tryptone, 0.5% yeast extract and 0.5% NaCl) unless mentioned otherwise. The minimal M9 medium (Miller, 1972), supplemented with 0.2% casamino acids, was used in

experiments when the expressed proteins were secreted into the growth medium and had to be purified.

M17 growth medium (Difco Laboratories, Detroit, MI, USA)) supplemented with 0.5% glucose (GM 17) was used for culturing L. lactis. Erythromycin was used at a concentration of 5 µg/ml. L. lactis is grown at 28°C without shaking.

The hybridomas and the myeloma cells were grown in RPMI 1640 (Gibco BRL, Bethesda, Md., USA) supplemented with 10% fetal calf serum, 0.3 mg/ml L-glutamine, 0.4 mM sodium pyruvate, 100 u/ml penicillin and 100 ng/ml streptomycin.

5. Adjuvants

For the first immunisation Ribi adjuvant (Ribi Immunochem Research Inc., Hamilton, MT, USA) was used. A complete dose of Ribi adjuvant contains 50 µg MPLA (monophosphoryl lipid A), 50 µg TDM (trehalose dicorynomycolate), 2% squalene and O.01% Tween 80.

For the second and third immunisation MPLA (Ribi Immunochem Research Inc., Hamilton, MT, USA) was used alone or mixed with an equal quantity of adjuvant peptide (Sigma Chemical Co., St. Louis, Mo., USA).

6. DNA manipulations

Restriction enzymes, DNA polymerases, T4 polynucleotide kinase and T4 DNA ligase (Boehringer, Mannheim, Germany; Gibco BRL, Bethesda, Md. USA, or New England Biolabs, Beverly, MA, USA) were used as recommended by the manufacturer. For analytical purposes, plasmid DNA was extracted according to Birnboim and Doly (1979). For preparative purposes, plasmid DNA was isolated according to Kahn et al. (1979). Restriction fragments of DNA were isolated by the Geneclean method according to Vogelstein and Gillespie (1979) and Struhl (1985). The required materials were purchased from Bio 101 (La Jolla, CA., USA). For the isolation of plasmid DNA out of L. lactis, a pretreatment of the bacteria is

necessary to weaken the cell wall. The bacterial pellet was resuspended in 50 µl TE (10 mM Tris-HCl pH 8 - 1 mM EDTA). Afterwards another 50 µl TE, supplemented with 10 mg/ml lysozyme (Boehringer, Mannheim, Germany) and 200 u/ml mutanolysin (Sigma Chemical Co., St. Louis, Mo., USA) was added. This mixture was incubated for 10 min at 37°C and then put on ice for 5 min. Further treatments are identical to those used for plasmid isolation out of E. coli.

For all constructions in L. lactis purified plasmid DNA (Qiagen, Hilden, Germany) were used. The DNA fragments were purified from agarose gels by using Qiaex II (Qiagen, Hilden, Germany).

7. PCR amplification

All PCR reactions were carried out following a basic protocol. In each reaction about 50 ng pure template and 50 pmol sense and anti-sense oligonucleotides (Life Technologies, Paisley, UK) were used. Two units $Vent_R$® DNA polymerase (New England Biolabs, Beverly, MA., USA) were added after heating of the samples to 94°C. The annealing temperature (T_a) was set, according to the composition of the primer, at about 7°C below the melting temperature (T_m). In these PCR amplifications the best results were obtained at 60°C. The synthesis of hbc and the fusion genes ipm2hbc and im2hbc, was carried out for 45 seconds at 72°C. The synthesis of the sequence, coding for the extracellular part of the M2 protein (cm2 and sm2), was left for 20 seconds at 72°C. A total of thirty amplification rounds were performed. The control reactions did not contain oligonucleotides. Three different concentration of $MgSO_4$ were used, 2, 3 and 4 mM. The PCR reaction that produced a significant amount of the expected fragment under the most stringent conditions (lowest Mg^{2+} concentration and highest T_m) was used for further cloning.

The C3d3 fragment was amplified from pSG5.C3d.YL with the oligonucleotides C3ds and C3da using

Pwo DNA Polymerase (Boehringer, Mannheim, Germany). The annealing temperature was set at 60°C and the synthesis was performed for 2 min at 72°C.

8. Ligation

The ligations for L. lactis were performed with Ready-To-Go™ T4 DNA Ligase (Pharmacia Biotech, Uppsala, Sweden). After incubation for 1h at 20°C, the mixture was extracted with phenol (Life Technologies, Paisley, UK) and chloroform/iso-amyl alcohol (24/1). The DNA was precipitated with see-DNA (Amersham International, Buckinghamshire, UK). The complete resuspended pellet was used for electroporation (Wells et al., 1993).

9. Protein purification media

All chromatography media were purchased from Pharmacia Biotech (Uppsala,Sweden), except CF11 cellulose, which was purchased from Whatman International Ltd. (Maidstone, UK).

10. Protein gel

Protein samples were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli, 1970. After electrophoresis, the proteins were fixed with 10% trichloroacetic acid and stained with 0.05% Coomassie brilliant blue R-250 in destain. Excess dye was removed by incubating the gel in destain (30% methanol - 7% acetic acid). The gel was soaked in 40% ethanol before it was dried between two sheets of permeable cellophane.

11. Western blot and dot blot

For immunological characterisation, proteins were electrophoretically transferred from a SDS-PAGE-gel onto a nitrocellulose membrane (pore diameter 0.45 μm, Schleicher & Schuell, Dassal, Germany) with a dry blotting apparatus (Plexi-labo, Gent, Belgium). The filter was blocked for at least 2h in PBS pH 7.4 (14.5 mM

phosphate buffer pH 7.4 - 150 mM NaCl) with 2.5% skim milk powder and 0.1 % Triton X-100 (blocking buffer). Incubation with the primary antibody, diluted in blocking buffer, was carried out at room temperature for 30 to 60 min. Excess of unbound antibody was removed by three washings with blocking buffer. The bound antibodies were detected with an alkaline phosphatase conjugated antibody of the appropriate specificity. Subsequently, the filter was washed two times with PBS pH 7.4 - 0.1% Triton X-100. A third washing step was carried out with substrate buffer (100 mM Tris-HCl pH 9.5 - 100 mM NaCl - 5 mM $MgCl_2$). The filter was then incubated in substrate buffer with 165 µg/ml nitro blue tetrazolium (NBT) and 165 µg/ml 5-bromo-4-chloro-3-indolylphosphate (BCIP) until a clear signal appeared. The blot was finally washed thoroughly with tap water and dried.

The dot blot analysis was carried out in a similar way as the Western blot, except that the proteins were not transferred through electrophoresis, but by filtering the samples through a nitrocellulose membrane.

12. ELISA

In every ELISA we used a 0.1 % casein solution for blocking and for the dilution of the antibodies used. The stock solution of casein (2.5%) was prepared as follows: 6.25 g casein powder was dissolved in 200 ml 300 mM NaOH by overnight stirring at 37°C. The pH was adjusted to 7.0 by adding 2N HCl. The final volume was brought to 250 ml (Nunc bulletin no. 7, December 1989). Sodium azide (0.02%) was added as a preservative.

Different ELISA's were developed to determine the concentration of Hepatitis B core or human ß2-microglobulin fusion protein. Microtiter plates (type II F96 maxisorp Nunc A/S, Roskilde, Denmark) were coated for 1.5 h at room temperature or overnight at 4°C with a 1/2 dilution series of samples containing IPM2HBcm or IPM2hB2Mm. On the same plate, a 1/2 dilution series of purified HBc or hB2M, respectively, starting from 2

μg/ml, was used as a standard. Between every incubation step, the plates were washed twice with tap water and once with PBS, pH 7.4 - 0.05% Triton X-100, except that after blocking, the plates were not washed. The microtiter plates were blocked with 0.1% casein solution for 2h at room temperature or at 4°C overnight. As primary antibody we used mouse anti-HBc or mouse anti-hB2M, respectively. The bound antibodies were detected with an alkaline phosphatase labelled anti-mouse IgG (γ chain specific) antibody. The incubation with antibody solution was carried out at room temperature for 1.5 h. Finally the microtiter plates were incubated for 1 h with substrate buffer (10% diethanolamine - 0.5 mM $MgCl_2$ - 0.02% NaN_3 pH 9.8) containing 1 mg/ml p-nitrophenyl phosphate. The absorbance was measured at 405 nm and the wave length of 490 nm was used for normalisation.

13. Preparation of polyclonal anti-M2

All mice, which had been immunised with IPM2HBcm and had survived the lethal challenge with m.a. A/PR/8/34 influenza A virus (see results, immunisation) were anaesthetised with 250 μl 25 mg/ml tribromoethanol (injected i.p.) and blood samples were taken by heart puncture. The serum was isolated as described hereinbelow. The crude serum gave a high background in Western blot, therefore an IgG fraction was prepared. The crude serum was filtered through a 0.45 μm filter (Millipore Millex-HV, Millipore, Bedford, MA, USA) and diluted 10 times in loading buffer (PBS - 10 mM EDTA pH 8). This mixture was loaded on an equilibrated Protein G Sepharose 4 Fast Flow column (ϕ = 1 cm, h = 8 cm), The bound IgG molecules were eluted with 100 mM glycine-HCl pH 2.7. Fractions of 1 ml were collected in tubes containing 50 μl 1 M Tris-HCl pH 9.5 to bring the pH to neutral.

The quantity of anti-M2 antibodies in the pooled peak fractions was 2.6 μg/ml. This was determined in an ELISA, comparable to the detection of anti-M2

antibodies in the serum of immunised mice. Mouse monoclonal anti-human ß2-microglobulin (Cymbus Bioscience, Southampton, UK) was used as a standard.

14. Serum preparation

Five blood samples were taken from every mouse: the pre-immune serum (a), the serum taken after the first (b), after the second (c) and after the third (d) immunisation, and the serum taken after challenge (e). This blood was incubated for 30 min at 37°C. The samples were then placed on ice for at least 1 hour and centrifuged two times 5 min at 16000 g in a microcentrifuge. The serum was isolated.
Equal volumes of sera obtained from different mice were pooled for the analysis of antibody production.

15. List of plasmids

15.1 E. coli

pATIPM2m1 : plasmid that contains the uninterrupted m2 gene from A/PR/8/34

pIPM2hB2Mm2s2 : plasmid for the expression of IPM2hB2Mm, with the correct amino terminus of M2

pPLcIPM2HBc : expression plasmid for IPM2HBc, with four amino acids between the initiating methionine and the amino terminus of M2e

pPLcIPM2HBcm : expression plasmid for IPM2HBcm, with the correct amino terminus of M2e. Sequence of M2 is derived from A/PR/8/34

pPLcIM2HBcm : expression plasmid for IM2HBcm, with the correct amino terminus of the universal M2

15.2 L. lactis

pT1TT : plasmid for the expression of TTFC

pT1PM2LTT : expression of IPM2TT, with leucine codons adapted for L. lactis. Sequence of M2e is derived from A/PR/8/34

pT1PM2LTTIL2 : expression of IPM2TT, with adapted leucine codons, in combination with mIL2

pT1PM2LTTIL6 : plasmid for the expression of IPM2TT, with adapted leucine codons, in combination with mIL6

pT1HBc : plasmid for the expression of HBc

pT1HBcIL2 : expression of HBc in combination with mIL2

pT1HBcIL6 : expression of HBc in combination with mIL6

pT1PM2HBc : plasmid for the expression of IPM2HBcm. Sequence of M2e is derived from A/PR/8/34

pT1PM2HBcIL2 : expression of IPM2HBcm in combination with mIL2

pT1PM2HBcIL6 : expression of IPM2HBcm in combination with mIL6

pT1M2HBc : plasmid for the expression of IM2HBcm, with the universal sequence for M2e

pT1M2HBcIL2 : expression of IM2HBcm in combination with mIL2

pT1M2HBcIL6 : expression of IM2HBcm in combination with mIL6

pT1PM2LHBc : plasmid for the expression of IPM2HBcm, with leucine codons adapted for L. lactis

pT1PM2LHBcIL2 : expression of IPM2HBcm, with adapted leucine codons, in combination with mIL2

pT1PM2LHBcIL6 : plasmid for the expression of IPM2HBc, with adapted leucine codons, in combination with mIL6

pT1M2LHBc : expression of IM2HBcm, with leucine codons adapted for L. lactis

pT1M2LHBcIL2 : expression of IM2HBcm, with adapted leucine codons, in combination with mIL2

pT1M2LHBcIL6 : expression of IM2HBcm, with adapted leucine codons, in combination with mIL6

pT1cM2L : plasmid for the expression of the cytoplasmic form of M2e, with leucine codons adapted for L. lactis.

pT1cM2LC3d : expression of cM2LC3d, with adapted leucine codons

pT1cM2LC3d3 : expression of cM2LC3d3 (with three consecutive C3d domains), with adapted leucine codons

pT1sM2LX : plasmid for the expression of the secreted and anchored form of M2e, with leucine codons adapted for L. lactis

pT1sM2LC3d : expression of sM2LC3d, with adapted leucine codons

pT1sM2LC3d3 : expression of sM2LC3d3 (with three consecutive C3d domains), with adapted leucine codons

EXPERIMENTAL SECTION

1. Construction of pATIPM2m

The RNA segment 7 of the influenza A virus, A/PR/8/34 (H1N1), was cloned by a procedure as described for RNA segment 4 in Min Jou et al., 1980. The resulting plasmid was named pATIPMA and is commercially available (LMBP catalogue 1992, no. 1774).

The mRNA of the M2 protein is not a colinear transcript of RNA segment 7. Indeed, an intron of 689 nucleotides had to be removed (Lamb et al., 1981).

In the plasmid pATIPMA, StuI cuts after the first nucleotide of the second exon (see figure 1a). This nucleotide was included in the synthetic oligonucleotides, that were used to code for the first exon. The synthetic first exon, encoding the amino-terminus of the mature M2 protein, was designed to contain a single stranded GATC overhang at its 5' end. This allowed us to make the connection to a preceding BamHI site in the vector pATIPMA and to replace the original first exon.

Furthermore codon usage was optimised for expression in E. coli.

Next, we introduced, by site-directed mutagenesis (Stanssens et al., 1989), a BclI site at the junction between the extracellular part and the membrane

anchoring region of the M2 protein (see figure 1 b). The amino acid sequence of the extracellular part was not changed. The resulting plasmid, pATIPM2m1, carries the uninterrupted m2 gene of A/PR/8/34.

2. Construction of IPM2hB2Mm

Parker and Wiley (1989) expressed human ß2-microglobulin in the periplasm of E. coli by making use of the plasmid p714. This plasmid contains the coding region for ß2-microglobulin preceded by the signal sequence of the outer membrane protein A of E. coli (OmpA-ss) (see figure 2a). The OmpA signal sequence is required for the translocation of the protein, to which this sequence is fused, to the periplasm. The signal sequence is cleaved off after transport. On plasmid p714, human ß2-microglobulin is under control of both the lipoprotein (lpp) and lacUV5 promoter. Addition of 1 mM IPTG to a mid-log phase culture leads to the production Of ß2-microglobulin.

The coding sequence of the extracellular part of the M2 protein, isolated as a BamHI-BclI fragment from pATIPM2m1, was inserted between the signal sequence of ompA and the human ß2-microglobulin (for details see figure 2a). Due to the construction, there were 9 additional nucleotides between the end of the ompa signal sequence and the beginning of the m2 fragment, which had to be removed (see figure 2b). This was done by looping out mutagenesis according to Nakamaye and Eckstein, 1986. As a result, the plasmid pIPM2hB2Mm2s2 was obtained.

3. Localisation of the IPM2hB2Mm

A freshly grown preculture of C3000 containing p714 or pIPM2hB2Mm2s2 was diluted 1/100 in LB with ampicillin. As described above, the hb2m and ipm2hb2mm genes are under control of the lacUV5 promoter. When the cultures reached a density of about $5.5x10^8$ bacteria/ml, they were divided in two and one half of each culture was induced with 1 mM IPTG. After 3 h induction, the bacteria

were harvested and fractionated. The periplasm of the bacteria was isolated by osmotic shock (Neu and Heppel, 1965). The remainder of the bacteria was sonicated (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and centrifuged for 10 min at 16000 g, to isolate the cytoplasm. The different samples were analysed on a SDS 15% PAGE-gel. Human B2M and the fusion protein IPM2hB2Mm were transported to the periplasm, whereas the precursors, where the signal sequence was still present, remained associated with the bacteria (data not shown). Determination of the amino-terminus of the mature IPM2hB2Mm (by courtesy of Dr. J. Vandekerckhove) by automated Edman degradation on a model 470A gas-phase sequencer coupled to a model 120A on-line phenylthiohydantoin amino acid analyser (Applied Biosystems, Foster City, CA., USA), demonstrated that the OmpA signal sequence was correctly cleaved off.

4. Purification of IPM2hB2Mm

The fusion protein IPM2hB2Mm could efficiently be expressed in the periplasm of E.coli. Whereas performing an osmotic shock is a critical procedure, especially on large volumes, Steidler et al. (1994) previously described an elegant system, based on the controlled expression of the Kil protein, to release periplasmic proteins in the growth medium.

The kil gene is present on a compatible plasmid under the tightly regulated P_L promoter, the leftward promoter of phage λ (Remaut et al, 1981). The plasmid pcI857K1 also carries the temperature sensitive repressor of the P_L promoter, cI857. The fusion protein IPM2hB2Mm is synthesised upon induction with 1 mM IPTG and at the end of the production phase, the culture is switched from 28°C to 42°C to induce Kil.

A fermentation (BioFlo IV fermentor, New Brunswick Scientific Co., Edison, N.J., USA) was carried out using the standard induction procedure described above. The culture was centrifuged in a contifuge 17RS

(Heraeus Instruments, Hanau, Germany) at 11000 g and the growth medium was isolated. The sodium chloride concentration of the growth medium was adjusted to 300 mM and buffered with 20 mM MES (2-(N-morpholino)ethanesulphonic acid), pH 6.5. This solution was loaded on a DEAE Sephacel column (ϕ = 5 cm, h = 6.5 cm), equilibrated with 20 mM MES, pH 6.5 - 300 mM NaCl. Under these conditions IPM2hB2Mm did not bind to the matrix. The ammonium sulphate concentration of the flow through was brought to 0.8 M with a 3.8 M $(NH_4)_2SO_4$ solution, pH 7. The mixture was loaded on a Phenyl Sepharose column (ϕ = 5 cm, h = 17 cm), equilibrated in 20 mM Tris-HCl, pH 7.5 0.8 M $(NH_4)_2SO_4$. A decreasing ammonium sulphate concentration gradient starting from 0.8 M and going to 0, did not release the bound fusion protein. This was achieved by eluting the column with a pH gradient from 20 mM Tris-HCl, pH 7.5 to 5 mM NaAc, pH 5.5. The peak fractions were pooled and diluted ten times in 20 mM diethylamine (DEA), pH 8.5.

The complete mixture was loaded on a Sepharose Q column (ϕ = 0.8 cm, h = 2.3 cm), equilibrated with 20 mM DEA, pH 8.5. The protein was eluted from the column with a salt gradient from 0 to 1 M. The peak fractions were pooled and loaded on a Sephacryl S-100 gel filtration column (ϕ = 1.5 cm, h 47 cm). Only one peak with the expected molecular weight of about 15 kDa was observed. This purified IPM2hB2Mm was used to immunise mice for preparing hybridomas, secreting monoclonal antibodies directed against the M2 protein.

5. Production of monoclonal antibodies to the M2 protein

Balb/c mice were immunised three times with 2.5 μg purified IPM2hB2Mm. For the first injection a complete dose of Ribi adjuvant was used. The second and third immunisation were performed in the presence of 50 μg MPLA. The injections were given with an interval of three weeks. Three days after the last immunisation, spleen cells were isolated and fused with myeloma cells

SP2/0-AG14 using standard protocols (Köhler and Milstein, 1975). Supernatants from different immunoglobulin producing cell clones were tested in ELISA and Western blot for reactivity against the other fusion protein IPM2HBcm (described further). The Hepatitis B core protein alone was used as a control to eliminate false positive clones. The isotype of the antibody was determined (Isostrip, Boehringer, Mannheim, Germany). Two different immunoglobulin subtypes that recognised the extracellular part of the M2 protein were obtained, an IgM and an IgG2a. Especially the IgG2a antibody was used in further experiments.

6. Expression of HBc and IPM2HBcm

Expression of proteins under control of the P_L promoter was performed by shifting an exponentially growing culture from 28°C to 42°C (Remaut et al., 1981). A saturated preculture of MC1061 [pcI857] containing the plasmid pPLc245 (control), pPLcA1 (carrying the hbc gene) or pPLcIPM2HBcm (containing the fusion gene ipm2hbc) respectively, was diluted 1/100 in LB medium (50 µg/ml kanamycin and 100 µg/ml ampicillin) and grown for about 4 h at 28°C under shaking. When the cultures reached a density of $4.5x10^8$ to $5.5x10^8$ bacteria/ml, they were split, one half was incubated for 4 h at 28°C, the other half was switched to 42°C. The bacteria were concentrated by centrifugation (2 min at 16000 g in a microcentrifuge).

The culture medium was removed and the bacteria were resuspended in TE buffer (10 mM Tris-HCl - 1 mM EDTA, pH 7.6). The bacteria were opened by sonication (Vibra cell, Sonics & Materials Inc., Danbury, Conn., USA) and the bacterial debris were pelleted for 10 min at 16000 g in a microcentrifuge. The supernatant was isolated and the pellet was resuspended in TE buffer. The samples were analysed on a SDS 12.5% PAGE-gel, in a Western blot and on a dot blot.

7. Large scale production of IPM2HBcm

The strain MC1061 [pcI857, pPLcIPM2HBcm] was grown in a BioFlo IV fermentor (New Brunswick Scientific Co., Edison, N.J., USA). When the culture reached a density of about $5.5x10^8$ cells/ml, the temperature was increased to 42°C. After three hours of induction, the culture was centrifuged in a contifuge 17RS (Heraeus Instruments, Hanau, Germany) at 11,000 g. The bacteria were collected and resuspended in a volume (in ml) buffer (50 mM Tris-HCl pH 8 - 150 mM NaCl - 5% glycerol with one protease inhibitor cocktail tablet (Complete™; Boehringer, Mannheim, Germany) per 25 ml) corresponding to two times the weight (in g) of the pelleted bacteria. This suspension was treated with 1 mg/ml lysozyme (freshly dissolved in 25 mM Tris-HCl pH 8) for half an hour on ice. Subsequently, the bacteria were lysed with 0.2% Triton X-100 in the presence of 25 mM EDTA, pH 8. After 30 min incubation on ice, the lysates were centrifuged for 1 h in a Sorvall SS-34 rotor (Du Pont Company, Wilmington, DE, USA) at 48000 g. The supernatant was removed and used for purification of IPM2HBcm.

8. Immunisation with IPM2HBcm

Balb/c mice were injected three times intraperitoneally with purified IPM2HBcm in the presence of adjuvant. Control mice received only PBS buffer, pH 7.4 and adjuvant. For the first immunisation half a dose of Ribi adjuvant was used. In the second and third injection we used 25 μg MPLA and 25 μg MDP.

Mice were immunised intranasally three times by applying a light ether anaesthesia, after which 50 microliter antigen solution in PBS buffer (containing either 10 microgram IPM2HBcm or IM2HBcm without any adjuvant) is put in the nostril.

9. Expression in L. lactis

Single colonies from L. lactis strain MG 1363, containing the plasmid pT1HBc, pT1PM2HBc or pT1M2HBc,

respectively or the derivatives with mIL2 (pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2) or mIL6 (pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6), were inoculated in 10 ml GM17E each. MG1363 [pTREX1] was used as control. The bacteria were grown for about 16 h at 28°C. The cells were collected by centrifugation at 2000 g for 20 min (Sorvall 11 RT6000 D). The growth medium was isolated and the bacteria were resuspended in 250 µl TE. Following resuspension, an additional 250 µl TE supplemented with 10 mg/ml lysozyme and 200 u/ml mutanolysin was added. This mixture was incubated for 10 min at 37°C and then put on ice for 5 min. Then 500 µl Laemmli sample buffer (100 mM Tris-HCl pH 6.8 - 5% SDS - 1.2M ß-mercaptoethanol - 0.008% bromophenol blue - 16% glycerol) was added and the samples were boiled for 5 min. An equivalent of 1 ml original culture volume, or 10^9 bacteria was analysed on a SDS 12.5% PAGE-gel. The production of mIL2 or mIL6 in the culture supernatant was evaluated in a bio-assay based on the proliferation of CTLL2-cells (mIL2, Gillis et al., 1978) or the proliferation of a B-cell hybridoma, 7TD1 (mIL6, Van Snick et al., 1986).

RESULTS

1. Construction of IPM2HBcm

The plasmid pPLcA1 (see figure 3a) contains the hepatitis b core (hbc) gene under control of the P_L promoter of bacteriophage λ (a gift from Dr. Nassal). The 346 bp NcoI-XbaI HBc fragment, isolated from pPLcA1, was inserted into the NcoI and XbaI opened pMa581, a derivative of pMa58. This plasmid was called pMaHBc. At the 5' end of the hepatitis B core, directly following the start codon, we introduced a BamHI site by site-directed mutagenesis (Stanssens et al., 1989), correctly positioned in the reading frame of HBc (for details see figure 3a and b). The resulting plasmid was named pMaHBcm. The information coding for the extracellular part of the M2 protein was cloned as a 72 bp BamHI-BclI fragment, derived from pATIPM2m1, into the BamHI opened

pMaHBcm, resulting in the vector pIPM2HBc. The hbc gene in the expression vector pPLcA1 was then replaced by the 418 bp NcoI-XbaI m2hbc fragment, creating pPLcIPM2HBc. Due to the construction, four amino acids extra were present between the first methionine and the start of the extracellular part of the M2 protein and had to be removed (see figure 3c). This was done by looping out mutagenesis (Deng and Nickolov, 1992). The resulting plasmid was named pPLcIPM2HBcm (see figure 3a and c).

2. Expression of the fusion protein

The plasmids pPLc245 (control), pPLcA1 (hbc gene) and pPLcIPM2HBcm (ipm2hbc gene) were transformed to MC1061 [pcI857]. After culture and induction the bacteria were lysed by sonication. The lysates were centrifuged and an aliquot of the supernatants was loaded on a SDS 12.5% PAGE-gel (see figure 4). The same fractions were also analysed by a Western blot. Two different monoclonal antibodies were used : an antibody specific for the Hepatitis B core protein and a monoclonal antibody (IgG2a) directed against the extracellular part of the M2 protein.

The monoclonal antibody against Hepatitis B core reveals two different bands (see figure 5A), one corresponding to the Hepatitis B core protein and the other to the fusion protein. The latter protein has a lower mobility, corresponding to the insertion of the extracellular domain of the M2 protein. The presence of the M2 fragment was confirmed by using the antibody specific for the extracellular part of the M2 protein (see figure 5B).

The N-terminal amino acid sequence of IPM2HBcm was determined (Dr. J. Vandekerckhove) by automated Edman degradation on a model 470A gas-phase sequencer coupled to a model 120A on-line phenylthiohydantoin amino acid analyser (Applied Biosystems, Foster City, CA., USA). This analysis revealed the N-terminal sequence Ser Leu Leu, which is exactly the same as the amino terminal

sequence of the M2 protein of the influenza A virus (figure 6). The first amino acid, methionine, was removed in E. coli. The amino-terminus of the fusion protein thus corresponds to that of the wild type M2 protein (table 1; Lamb et al., 1985).

Hepatitis B core, also when expressed in E. coli, spontaneously associates to form particles, indistinguishable from the viral core particles circulating in the blood of Hepatitis B infected patients (Cohen and Richmond, 1982). Clarke and co-workers (1987) showed that a peptide inserted at the amino terminus of the Hepatitis B core protein could be detected at the surface of the particle.

Electron micrographs (Dr. G. Engler) showed that the IPM2HBcm fusion protein was able to form similar particles. To investigate whether the insertion of the extracellular part of the M2 protein resulted in the surface localisation of this fragment, soluble fractions, containing HBc or IPM2HBcm, were loaded on a nitrocellulose membrane in a dot blot. The dot blots were treated with a monoclonal antibody directed against HBc or against M2. Figure 7 clearly shows a signal in the soluble pPLcIPM2HBcm fraction, when revealed with the antibody directed against the M2 protein (panel B). Since the soluble fraction is loaded in a native state onto the nitrocellulose membrane, we conclude that the epitope is located at the surface of the Hepatitis B core particle.

3. Purification of IPM2HBcm

The bacterial lysates were prepared as described in Materials and methods. The concentration of Tris-HCl pH 8 and NaCl were adjusted to 20 mM and 50 mM respectively. This mixture was loaded on a DEAE Sepharose column (ϕ = 2.5 cm, h = 5.5 cm), equilibrated with 20 mM Tris-HCl, pH 8-50 mM NaCl. The fusion protein was not retained on the column. To the flow through 3.8 M $(NH_4)_2SO_4$, pH 7 was added to a final concentration of 1.2 M. This mixture was incubated under stirring in the cold

room during 16h. The precipitate was removed over a CF11 cellulose column (ϕ = 2.5 cm, h = 3.5 cm). The column was eluted with PBS, pH 7.4. The eluate of about 50 ml was concentrated in a Centiprep 30 (Amicon Corporation, Danvers, Ill., USA) to 5 ml and loaded on a Sephacryl S-300 column (ϕ = 2.5 cm, h = 91 cm), which was equilibrated with PBS, pH 7.4. The peak fractions were pooled and the concentration of IPM2HBcm was determined in an ELISA, The LPS content was assayed (LAL Coatest® Endotoxin purchased from Endosafe Inc., Charleston, SC., USA) and was sufficiently low (5 to 9 ng/50 μg IPM2HBcm) not to interfere with immunisation.

4. Immunisation

The purified preparation of IPM2HBcm particles was used to immunise 7 weeks old female Balb/c mice. Four different groups of 12 mice were evaluated. The first group received 50 μg IPM2HBcm, the second 10 μg, the third 5 μg and the fourth a control group, only received buffer with adjuvant. A total of three injections were given with the appropriate adjuvant. The injections were administered with three weeks interval. Three weeks after the last inoculation, the mice were challenged with 5 LD_{50} m.a. A/PR/8/34. The virus was administered intranasally in a total volume of 50 μl after ether anaesthesia. Morbidity was followed by measuring rectal temperature (figure 8 A1) and weight (figure 8 A2) every other day.

All mice immunised with IPM2HBcm showed a significant degree of protection against the following influenza challenge. Depending on the administered dose, 9 to 11 mice out of 12 survived the influenza infection, versus only 2 out of 11 for the control group (see figure 8B).

5. Analysis of the serum samples

One day prior to the first (bleeding a) and two weeks after every injection (bleeding b, c and d) blood samples were taken. Three weeks after the challenge, when

the mice had recovered sufficiently from the influenza infection, a last blood sample (e) was taken. The serum was analysed in an ELISA (see Materials and methods) to identify IgG antibodies directed towards the extracellular part of the M2 protein. To do so, we made use of the other fusion protein, IPM2hB2Mm. One half of the microtiter plate was coated with human ß2-microglobulin, the other half was coated with the fusion protein IPM2hB2Mm, both as unpurified culture supernatant. The concentration of IPM2hB2Mm used was 1 µg/ml. The same concentration of total protein was used in both set ups. Therefore, the hB2M content of the culture supernatant of bacteria expressing hB2M had to be adjusted to 1 µg/ml by adding purified hB2M (Sigma Chemical Co., St. Louis, Mo., USA). Dilution series (1/3) of the different serum samples, starting from 1/50, were loaded on the hB2M and IPM2hB2Mm, coated wells. The ELISA was further developed as described in Materials and methods.

To obtain the value for the specific reactivity towards the extracellular part of the M2 protein, the absorbance of hB2M at a given dilution was subtracted from the absorbance of IPM2hB2Mm of the corresponding dilution. Figure 9 clearly demonstrates a high antibody response to the extracellular part of the M2 protein, in the mice which received three injections with the vaccine. The titer in the serum was further increased after the challenge.

6. Construction of IM2HBcm

It is the aim of the present invention to make a universal vaccine against influenza A viruses. In the vaccination studies described above, we showed protection against the influenza virus from which the original M2 sequence was derived, A/PR/8/34 (homologous protection). The extracellular part of the M2 protein from this virus differs from most other viruses sequenced to date, by only one amino acid (see table 1). Therefore, a construct

was made in which the glycine at position 20 was changed to aspartic acid.

To do so we made use of an intermediate vector in the construction of pPLcIPM2HBcm, pMaIPM2HBc2 (see figure 3a). The plasmid pMaIPM2HBc2 does not yet contain the mutated m2 (deletion of 12 extra nucleotides) fragment, which starts at the first mature codon of the M2 protein. Therefore this fragment was isolated from pPLcIPM2HBcm by cutting with SgrAI and EcoRI. This 499 bp SgrAI-EcoRI fragment was cloned into the SgrAI and EcoRI opened vector pMaIPM2HBc2, which resulted in the construction of pMaIPM2HBc3 (see figure 10).

By site-directed mutagenesis according to Deng and Nickoloff (1992) the sequence of the extracellular part of the M2 protein was changed to the more universal M2 sequence (Gly20 -> Asp). The new plasmid was called pIM2HBcm. The sequence was determined on a model 373A sequencer (Applied Biosystems, Foster city, CA., USA) and shown to contain the desired mutation. The mutated m2 fragment was isolated from pIM2HBcm as a 499 bp SgrAI-EcoRI fragment and reintroduced into the expression vector pPLcIPM2HBcm, opened with SgrAI and EcoRI, to create pPLcIM2HBcm.

7. Expression of IM2HBcm

Strain MC1061 [pcI857] containing respectively pPLc245, pPLcA1, pPLcIPM2HBcm or pPLcIM2HBcm was cultured as described in the Experimental Section. The bacteria were collected and opened by sonication. The soluble fraction was isolated and the concentration of Hepatitis B core protein or the derived fusion proteins was determined in an ELISA. A soluble fraction containing 5 µg HBc or I(P)M2HBcm was analysed on a SDS 12.5% PAGE-gel (see figure 11). The same fractions were also analysed in a Western blot (see figure 12). The proteins of interest were detected with an antibody directed against the Hepatitis B core protein or with the monoclonal antibody specific for the extracellular part of the M2 protein. It

can be concluded that the new fusion protein, IM2HBcm, is expressed as efficiently as IPM2HBcm. Moreover the amino acid change in the extracellular part of the M2 protein (Gly20 --> Asp) has no effect on the binding of the monoclonal anti-M2 antibody.

8. Immunisation against heterologous challenge

A similar procedure as described in point 4 was used to test the efficiency of IPM2HBcm and IM2HBcm to protect mice versus heterologous challenge with influenza. 10 microgram of IPM2HBcm or IM2HBcm (purified in an identical way as IPM2HBcm) was used for immunisation. The mice were challenged with 30 HAU X-47.

All mice immunised showed a significant degree of protection against the heterologous challenge. 8 (in case of IPM2HBcm, $p<0.05$) or 12 (in case of IM2HBcm, $p<0.0001$) mice out of 12 survived the influenza infection, versus only 2 out of 11 in the control group (figure 8C).

To test the effect of intranasal administration, the same procedure was followed, but instead of the intraperitoneal injection, the antigen was administered intranasally. Also in this case, the protection is evident: 12 (in case of IPM2HBcm, $p<0.0001$) or 11 (in case of IM2HBcm, $p<0.001$) mice out of 12 survived the influenza infection, versus 2 out of 11 in the control group (figure 8D).

9. Construction of vectors for the expression of M2-HBc fusion proteins in L. lactis

The plasmid pTREX1 (Wells and Schofield, 1996) was used to express the Hepatitis B core protein and two M2-HBc fusion proteins, IPM2HBcm and IM2HBcm, in Lactococcus lactis. This plasmid has a constitutive L. lactis chromosomal promoter, P1, which is followed by the translation initiation region of the E. coli bacteriophage T7 gene 10 (Wells and Schofield, 1996). The transcription terminator is derived from T7 RNA

polymerase. The plasmid pTREX1 also carries two genes for resistance to erythromycin.

The expression plasmid, pTREX1, was cut with SphI, leaving a 3'CATG extension which was removed with Klenow DNA polymerase. The removed nucleotides were included in the sense linker for PCR amplification of the different genes. The linearised vector was then cut with BamHI and treated with CIP (calf intestine phosphatase, Boehringer, Mannheim, Germany).

The genes hbc, ipm2hbc and im2hbc were amplified by PCR (see Materials and methods). The anti-sense linker (HBca) was identical in all amplifications and provided a SpeI and a BclI site after the stop codon (see figure 13). For the amplification of ipm2hbc and im2hbc the same sense oligonucleotide (M2s) could be used, since the mutation Gly → Asp in the extracellular part of the M2 protein is located further downstream.

The amplification of hbc from pPLcA1 was only possible after the vector had been linearised with ScaI. The amplification reaction that produced a sufficient amount of fragment, under the most stringent conditions, was used for further cloning. The amplified fragment, hbc, ipm2hbc or im2hbc, was cut with BclI, phosphorylated with T4 polynucleotide kinase and inserted in the SphI and BamHI opened pTREX1 (see figure 14). The new plasmids were called pT1HBc, pT1PM2HBc (in which the extracellular part of the M2 protein is derived from the virus A/PR/8/34) and pT1M2HBc (in which the sequence of the extracellular part of the M2 protein corresponds to the type present in nearly all human influenza A viruses sequenced to date), respectively. The sequence of the inserted fragment was determined on a model 373A sequencer (Applied Biosystems, Foster City, CA., USA) and shown to be correct.

In view of using Lactococcus lactis as an improved vaccine delivery vehicle, two murine cytokines, interleukin 2 (mIL2) and interleukin 6 (mIL6) were inserted as second cistrons in the same operon as the

antigen. In that way we could obtain bacteria expressing the antigen, e.g. IM2HBcm, together with secreted murine interleukin 2 or 6. To obtain secretion of the interleukins into the growth medium, they were fused in frame to the lactococcal usp45 secretion signal peptide (van Asseldonk et al., 1990). The plasmids pT1HBc, pT1PM2HBc and pT1M2HBc were cut with SpeI and treated with CIP. The murine interleukin 2 gene was isolated as a 572 bp XbaI-SpeI fragment from plasmid pL2MIL2 (Steidler et al., 1995). This fragment was inserted into the SpeI opened pT1HBc, pT1PM2HBc and pT1M2HBc giving rise to pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2, respectively. In an analogous way the murine interleukin 6 gene was isolated as a 687 bp XbaI-SpeI fragment from pL2MIL6 (Steidler et al., 1996) and inserted into the SpeI opened vectors, pT1HBc, pT1PM2HBc and pT1M2HBc, to create pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6, respectively.

10. Expression of HBc and M2HBc in L. lactis

Lactoccoccus lactis strain MG1363 (Gasson, 1983) containing the plasmids for the expression of the antigen alone (pT1HBc, pT1PM2HBc and pT1M2HBc) or in combination with mouse interleukin 2 (pT1HBcIL2, pT1PM2HBcIL2 and pT1M2HBcIL2) or mouse interleukin 6 (pT1HBcIL6, pT1PM2HBcIL6 and pT1M2HBcIL6) were cultured as described in Materials and methods. MG1363 [pTREX1] was used as control.

An equivalent of 10^9 bacteria was analysed by SDS 12.5% PAGE. The expression of the Hepatitis B core and the M2-HBc fusion proteins were analysed by Western immunoblotting (see figure 15) carried out as described in Materials and methods. The expression of IM2HBc in MG1363 [pT1M2HBcIL6] was not as high as in the other constructs. By screening different colonies a clone could be isolated with comparable expression levels.

The production and secretion of interleukins into the growth medium was analysed in a biological assay. The biological activity of mIL2 was assayed by the

proliferation of a T-cell line, CTLL2 (Gillis et al., 1978) as compared to a human IL2 standard. The biological activity of mIL6 was measured by the proliferation of a B-cell hybridoma, 7TD1 (Van Snick et al., 1986). Table 2 gives an overview of the level of interleukin 2 and 6 per ml culture medium produced by the different expression plasmids. The supernatant of cultures producing mIL6 did not lead to proliferation in a mIL2 assay and vice versa.

Table 2

Plasmid	mIL2 production	mIL6 production
pT1HBcIL2	410 ng/ml	-
pT1PM2HBcIL2	481 ng/ml	-
pT1M2HBcIL2	359 ng/ml	-
pT1HBcIL6	-	1020 ng/ml
pT1PM2HBcIL6	-	772 ng/ml
pT1M2HBcIL6	-	802 ng/ml

11. Adaptation of the coding sequence of M2e to expression in L. lactis

Since the two fusion proteins, IPM2HBcm and IM2HBcm could hardly be detected in a Western blot, we tried to augment the production of these two fusion proteins by adapting the codon usage of the extracellular part of the M2 protein to L. lactis (van de Guchte et al., 1992).

At the 5' end of the extracellular part of the M2 protein we observed two consecutive leucine codons (CUG CUG) that were optimal for expression in E coli (68%), but poor for translation in L. lactis (8%, percentages described in van de Guchte et al., 1992). Therefore these codons were changed to UUA. The genes for ipm2hbc and im2hbc were amplified by PCR from respectively pPLcIPM2HBcm or pPLcIM2HBcm, with a new sense primer, M2Ls, containing the two changed leucine

codons (see figure 13). As anti-sense primer we used again HBca (see figure 13). The cloning of the genes was analogous as depicted in figure 14. The vectors so created were called pT1PM2LHBc and pT1M2LHBc.

The expression level of the mutated M2HBc proteins, compared to the original fusion proteins, was analysed in a Western blot (see figure 16). The expression level of the M2HBc fusion proteins with the L. lactis adapted leucine codons, was indeed much higher. It is concluded that the adaptation of codon usage to the L. lactis translation machinery, has a positive effect on the level of protein produced. In a similar way as described above, the murine interleukin 6 gene was inserted into pT1PM2LHBc and pT1M2LHBc, giving rise to pT1PM2LHBcIL6 and pT1M2LHBcIL6, respectively.

12. Construction of M2C3d in Lactococcus lactis

A second carrier protein, C3d, is also an attractive molecule for the presentation of the extracellular part of the M2 protein. Dempsey et al. (1996) demonstrated that the attachment of an antigen to three consecutive C3d molecules, was much more efficient in producing a high antibody response than the antigen administered in complete Freund's adjuvant.

The universal sequence of the extracellular part of the M2 protein, with the adapted leucine codons, is used for making a fusion to the amino-terminus of the first C3d molecule. The coding sequence for three different fusion proteins are constructed. In the first situation the M2C3d3 fusion protein is expressed in the cytoplasm of L. lactis (cM2C3d3), similar to the M2HBc fusion proteins. In the second case the M2C3d3 protein is secreted into the growth medium by making an in frame fusion to the usp45-signal sequence (sM2C3d3) and the last construct, which is a derivative of the secreted form, contains in addition an anchor sequence (spaX) after the last C3d molecule to attach the fusion protein covalently in the cell wall (sM2C3d3X).

The amplified C3d3 fragment was first subcloned in a derivative of pUC18, namely pUCB/S. pUC18 was linearised with HindII and a BglII linker was inserted. The resulting plasmid was then opened with SmaI and a SpeI linker was inserted, resulting in the plasmid pUCB/S (see figure 18). Three succeeding copies of C3d were amplified from pSG5.C3d3.YL (a gift from Dr. D. Fearon) by PCR with the oligonucleotides C3ds and C3da (see figure 17). This amplified fragment was cut with BglII and SpeI. The resulting 2830 bp BglII-SpeI fragment was cloned into the BglII and SpeI opened vector pUCB/S (see figure 18). The genes cm2 and sm2 were amplified by PCR. For the amplification of cm2 we used the sense oligonucleotide M2Ls (see figure 13) and the anti-sense linker M2Ca, which carried for our purposes a BamHI site in the correct reading frame (see figure 17). The same anti-sense linker was used for the amplification of sm2. The sense oligonucleotide for the amplification of sm2, M2LSs, started at the first codon of the mature M2 protein.

For the synthesis of the cytoplasmic form of M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pTREX1 analogous as the m2hbc gene described above (see also figure 18). The amplified cm2 fragment was cut with BamH I (77 bp), phosphorylated with T4 polynucleotide kinase and inserted in the Sph I and BamH I opened pTREX1, creating pT1cM2L. For the synthesis of the secreted and anchored form of M2C3d3, the information coding for the extracellular part of the M2 protein was inserted into pT1NX. The vector pT1NX carries the usp45-signal sequence (usp45-ss) and the anchor sequence derived from Staphylococcus aureus protein A (spaX). The plasmid pT1NX was cut with Nae I, correctly positioned at the end of the usp45-ss and BamH I. The amplified fragment, sm2, was cut with BamH I and phosphorylated with T4 polynucleotide kinase. This 73 bp sm2 fragment was inserted into the Nae I and BamH I opened pT1NX, resulting in the plasmid pT1sM2LX (see

figure 18). One single C3d fragment, isolated from pUCC3d, can then be inserted into the BamH I site at the end of the cm2 or sm2 sequence. Afterwards one or two additional C3d copies will be inserted.

13. Construction of M2TTFC in Lactococcus lactis

A third carrier protein, tetanus toxin fragment C (TTFC), can also be used. TTFC has already been expressed in L. lactis under control of the P1 promoter, pT1TT (Wells and Schofield, 1996). L. lactis expressing TTFC in combination with mIL2 or mIL6 to raise the antibody production, was successfully used in immunisation experiments (Patent GB 9521568.7). As positive control for analysis of antibody response in the present immunisation experiments with L. lactis expressing I(P)M2HBcm, a fusion was made between the extracellular part of the M2 protein and the amino terminus of TTFC.

The ttfc gene was amplified by PCR (see Materials and methods) from pT1TT. The sense oligonucleotide (TTFCs) provided a BamH I site, positioned in the correct reading frame, before the second codon of ttfc, corresponding to threonine. The anti-sense linker (TTFCa) provided a Spe I and a BamH I site after the stop codon (see figure 19). The amplification reaction that produced a sufficient amount of fragment, under the most stringent conditions, was used for further cloning (see Materials and methods). The amplified ttfc fragment was cut with BamH I, phosphorylated with T4 polynucleotide kinase and inserted in the Bcl I opened pATIPM2m1 (see figure 20). This plasmid construct was called pATIPM2TT. From this plasmid the m2ttfc gene was amplified by PCR (see Materials and methods) with M2Ls and TTFCa (see figure 19). The amplified m2ttfc fragment was cut with BamH I, phosphorylated with T4 polynucleotide kinase and inserted in the Sph I and BamH I opened pTREX1 (see figure 20). The new plasmid was called, pT1PM2LTT. In this construct

the extracellular part of the M2 protein is derived from the virus A/PR/8/34, with the two leucine codons adapted for use in L. lactis. The sequence of the inserted fragment was determined on a model 373A sequencer (Applied Biosystems, Foster City, CA., USA) and shown to be correct.

The murine interleukin genes, mIL2 and mIL6, were inserted in the same operon as m2ttfc. The murine interleukin 2 gene was isolated as a 572 bp Xba I - Spe I fragment from plasmid pL2MIL2 (Steidler et al., 1995). This fragment was inserted into the Spe I opened pT1PM2LTT giving rise to pT1PM2LTTIL2 (see figure 20). In an analogous way the murine interleukin 6 gene was isolated as a 687 bp Xba I - Spe I fragment from pL2MIL6 (Steidler et al., 1996) and inserted into the Spe I opened vector pT1PM2LTT to create pT1PM2LTTIL6 (see figure 20).

14. Expression of TTFC and M2TTFC in L. lactis

Lactoccoccus lactis strain MG1363 (Gasson, 1983) containing the plasmids for the expression of the antigen alone (pT1PM2LTT) or in combination with mouse interleukin 2 (pT1PM2LTTIL2) or mouse interleukin 6 (pT1PM2LTTIL6) were cultured as described in Materials and methods. MG1363 [pT1TT] was used as a control. An equivalent of 10^9 bacteria was analysed by SDS 10% PAGE. The expression of the IPM2TTFC fusion protein was analysed by Western immunoblotting (see figure 21) carried out as described in Materials and methods. The production and secretion of interleukins into the growth medium was analysed by a biological assay. L. lactis [pT1PM2LTTIL2] produced about 500 ng/ml mIL2 and L. lactis [pT1PM2LTTIL6] about 1 μg/ml mIL6. These results are comparable with the expression levels obtained with I(P)M2HBcm in combination with the two interleukins.

DISCUSSION

The present example describes several systems for the presentation of the highly conserved extracellular part of the influenza A virus M2 protein to the immune system. The M2 fragment was fused to the amino terminus of the carrier protein in order to retain a free N-terminus of the M2-domain and in this way mimic the wild type structure of the M2 protein. The first fusion protein, M2 linked to human ß2-microglobulin (IPM2hB2Mm), was used to produce monoclonal antibodies. A second fusion protein, M2 linked to Hepatitis B core protein (IPM2HBcm) was used for vaccination studies. Both proteins could also be used in the detection of a specific antibody response against the extracellular part of the M2 protein, since a correction has to be made for antibodies directed against the carrier protein, which are also produced during the immunisation process.

The vaccination studies with IPM2HBcm showed that the administered dose in the range that was used, was apparently not a very critical parameter for obtaining protection, as a dose ranging from 5 to 50 μg protected the mice, although the immunised mice still showed a high morbidity. This may have been due to the high dose of virus (5 LD_{50}) that was used for the challenge in order to obtain a clear-cut result for the degree of protection. In a natural influenza infection the number of infecting virus particles is much lower, so that it can be assumed that the morbidity would decrease accordingly.

Analysis of the serum of immunised mice showed a substantial antibody response towards the extracellular part of the M2 protein, especially after viral challenge. This latter, high response can be due to another way of administration, intraperitoneal versus intranasal. Or it can be explained on the basis of a more complete defense mechanism against the incoming virus.

Slepushkin et al. (1995) described a vaccination strategy, based on a membrane extract

containing the natural complete M2 protein for homologous and heterologous virus challenge. But they used a very strong adjuvant, incomplete Freund's, which is not appropriate for medical use.

In contrast, the M2 extracellular domain fusions of the invention described here can be obtained in a pure form (at least 95% purity), and can be administered in combination with safe adjuvants. A high degree of protection was obtained, despite the fact that the challenge was fairly severe. In view of the almost invariant sequence of the M2 extracellular domain (see table 1 which shows an overview of the amino acid sequences of the extracellular domain of the influenza A M2 protein) it may be expected that the protection achieved will be similar against all human influenza A strains known so far.

The vaccine may be further improved by the inclusion of an influenza specific T helper epitope as well as a CTL epitope into the fusion protein, for example internally or linked to the C-terminus of the Hepatitis B core protein. Other immunisation routes are possible as well, for example intraperitoneal versus intranasal.

Besides the gram negative organism, E. coli, also L. lactis was used, a gram positive organism, for the expression of the M2HBcm fusion proteins. In L. lactis it is not necessary to purify the expressed fusion protein. The bacteria can be administered directly either intranasally or orally.

A third promising carrier protein is also described, namely the third complement protein fragment d (C3d) (Dempsey et al., 1996). In a preferred construction, three copies of the C3d protein are preceded by the extracellular domain of the M2 protein. This M2C3d3 fusion protein can be expressed either intracellular, anchored in the cell wall or secreted into the growth medium by genetic fusion to appropriate regulatory sequences.

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CLAIMS

1. Influenza antigen, comprising a fusion product of at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and a presenting carrier.

2. Influenza antigen, wherein the presenting carrier is a presenting (poly)peptide.

3. Influenza antigen, wherein the presenting carrier is a non-peptidic structure, such as glycans, peptide mimetics, synthetic polymers.

4. Influenza antigen as claimed in claims 1-3 further comprising an additional domain for enhancing the cellular immune response immunogenicity of the antigen.

5. Influenza antigen as claimed in claims 1-4, wherein the conserved influenza membrane protein is the M2 membrane protein.

6. Influenza antigen as claimed in claim 5, wherein the M2 membrane protein originates from influenza A virus.

7. Influenza antigen as claimed in claims 1-6, wherein the presenting (poly)peptide is selected from the hepatitis B core protein, one or more C3d domains, tetanus toxin fragment C.

8. Influenza antigen as claimed in claims 1-7, wherein the antigen consists of Lactococci cells expressing the fusion product in or on their cell membrane.

9. Influenza antigen as claimed in claims 1-8, wherein the functional fragment of the conserved influenza membrane protein is a fragment that is capable of eliciting a statistically significant higher immunoprotection when administered in an immunoprotective dose to test members of a species than is found in control members of the same species not receiving the functional fragment.

10. Influenza antigen as claimed in claims 1-9, wherein the additional domain is an influenza specific T helper cell epitope or cytotoxic T cell epitope.

11. Influenza antigen as claimed in claims 1-10, obtainable by preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation regulatory sequences, bringing this gene construct in a suitable acceptor cell, effecting expression of the gene construct in the acceptor cell and optionally isolating the antigen from the acceptor cell or its culture medium.

12. Influenza antigen as claimed in claim 11, wherein the coding sequence for the extracellular part of a conserved influenza membrane protein consists of a coding sequence for the extracellular part of the M2 protein of the influenza A virus or a functional fragment thereof and the coding sequence for the presenting (poly)peptide is selected from coding sequences for hepatitis B core protein, one or more C3d domains, tetanus toxin fragment C.

13. Influenza antigen as claimed in claims 1-12, comprising the amino acids 2 to 24 of the M2 protein of influenza A virus, or modified versions thereof not substantially altering the tertiary structure of this part of the protein and hepatitis B core protein and/or one or more C3d domains.

14. Influenza antigen as claimed in claims 1-13 for use in the preparation of a vaccine against influenza for humans and animals.

15. Influenza antigen as claimed in claims 1-14 for use in the preparation of a vaccine against influenza A for humans and animals.

16. Vaccine against influenza, comprising at least an antigen as claimed in claims 1-15, optionally in the presence of one or more excipients.

17. Vaccine as claimed in claim 16, wherein the antigen is in isolated form.

18. Vaccine as claimed in claim 16, wherein the antigen is part of a membrane fragment.

19. Vaccine as claimed in claim 16, wherein the antigen is anchored in the membrane of an acceptor cell expressing the antigen.

20. Vaccine as claimed in claim 16, wherein the antigen consists of Lactococci cells expressing the fusion product in or on their cell envelope.

21. Vaccine as claimed in claims 16-20, further comprising one or more other influenza antigens, for example selected from haemagglutinin, neuraminidase nucleoprotein and/or native M2.

22. Use of an antigen as claimed in claims 1-13 for the preparation of a vaccine against influenza.

23. Method of preparing an antigen as claimed in claims 1-15, comprising the steps of:

a) preparing a gene construct comprising a coding sequence for at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and at least one coding sequence for a presenting (poly)peptide operably linked thereto, optionally in the presence of suitable transcription and/or translation regulatory sequences,

b) bringing this gene construct in a suitable acceptor cell,

c) effecting expression of the gene construct in the acceptor cell, and

d) optionally isolating the antigen from the acceptor cell or its culture medium.

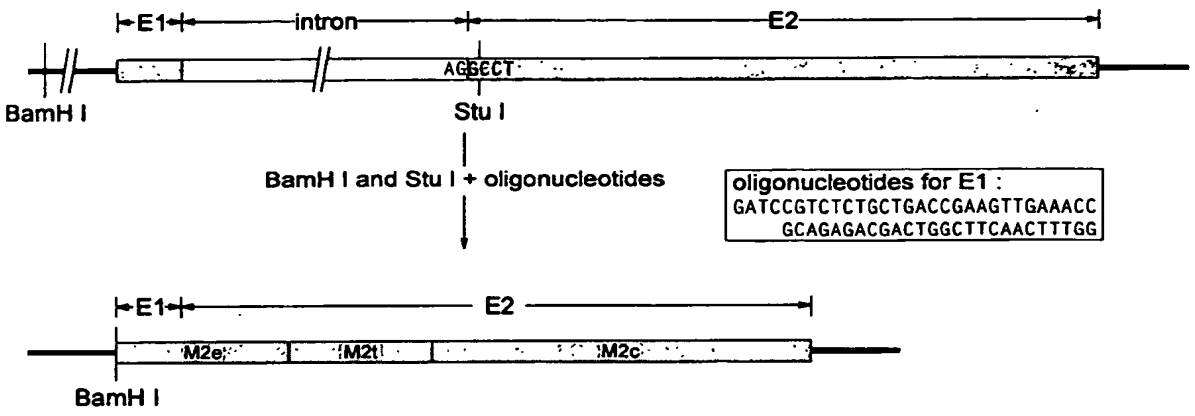
24. Acceptor cell, expressing an antigen as claimed in claims 1-15.

25. Acceptor cell as claimed in claim 24, wherein the cells are Lactococcus cells.

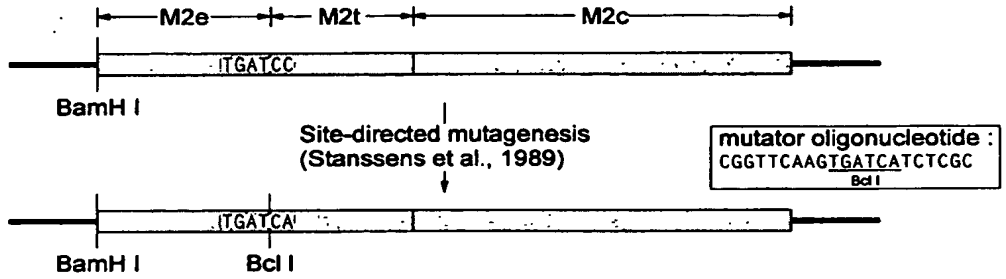
H 05.08.97

Figure 1

A



B



C

	2	3	4	5	6	7	8	9	10	11
Nucleotide sequence :	TCT	CTG	CTG	ACC	GAA	GTT	GAA	ACC	CCT	ATC
Amino acid sequence :	Ser	Leu	Leu	Thr	Glu	Val	Glu	Thr	Pro	Ile

12	13	14	15	16	17	18	19	20	21	22	23	24
AGA	AAC	GAA	TGG	GGG	TGC	AGA	TGC	AAC	GGT	TCA	AGT	GAT
Arg	Asn	Glu	Trp	Gly	Cys	Arg	Cys	Asn	Gly	Ser	Ser	Asp

figure2a1

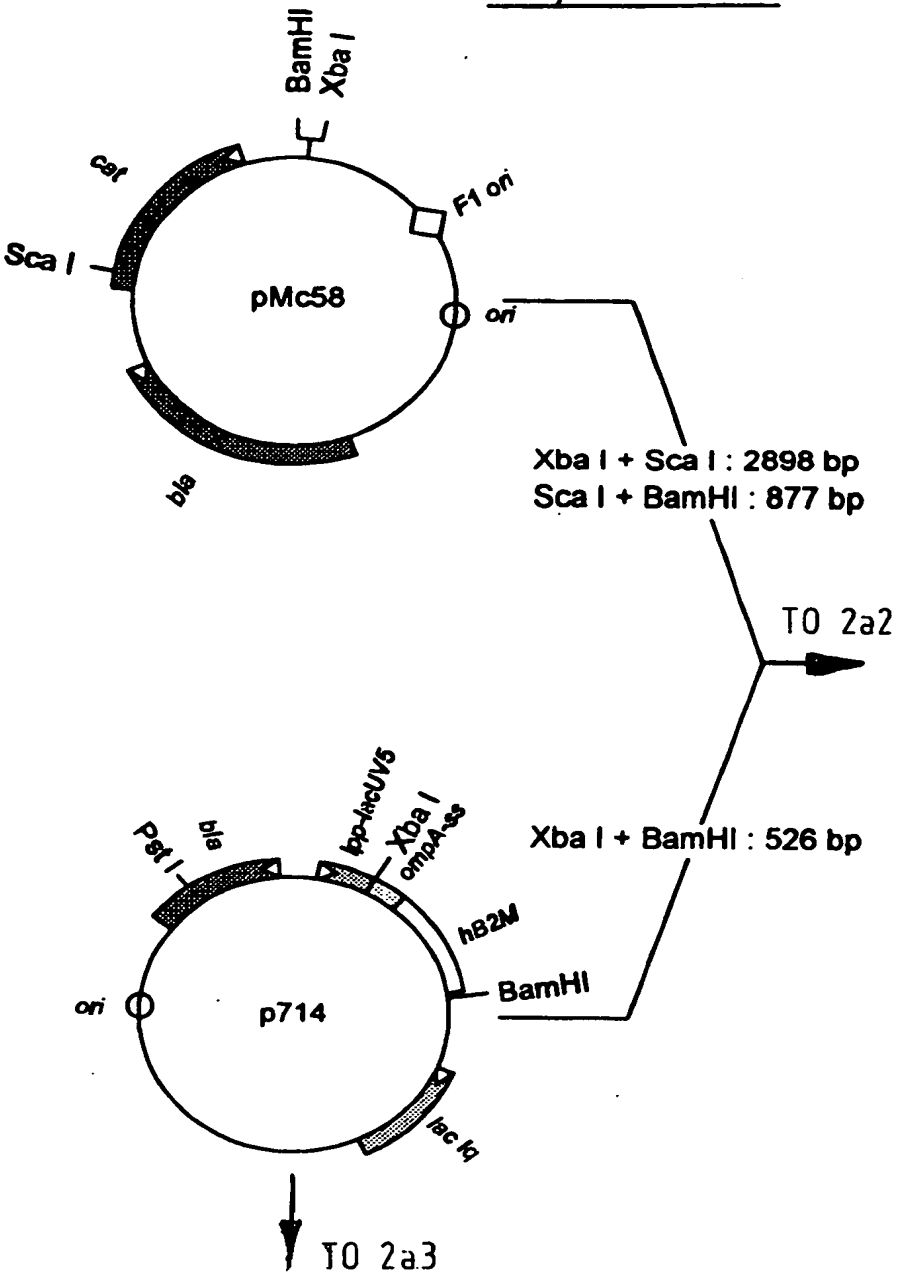
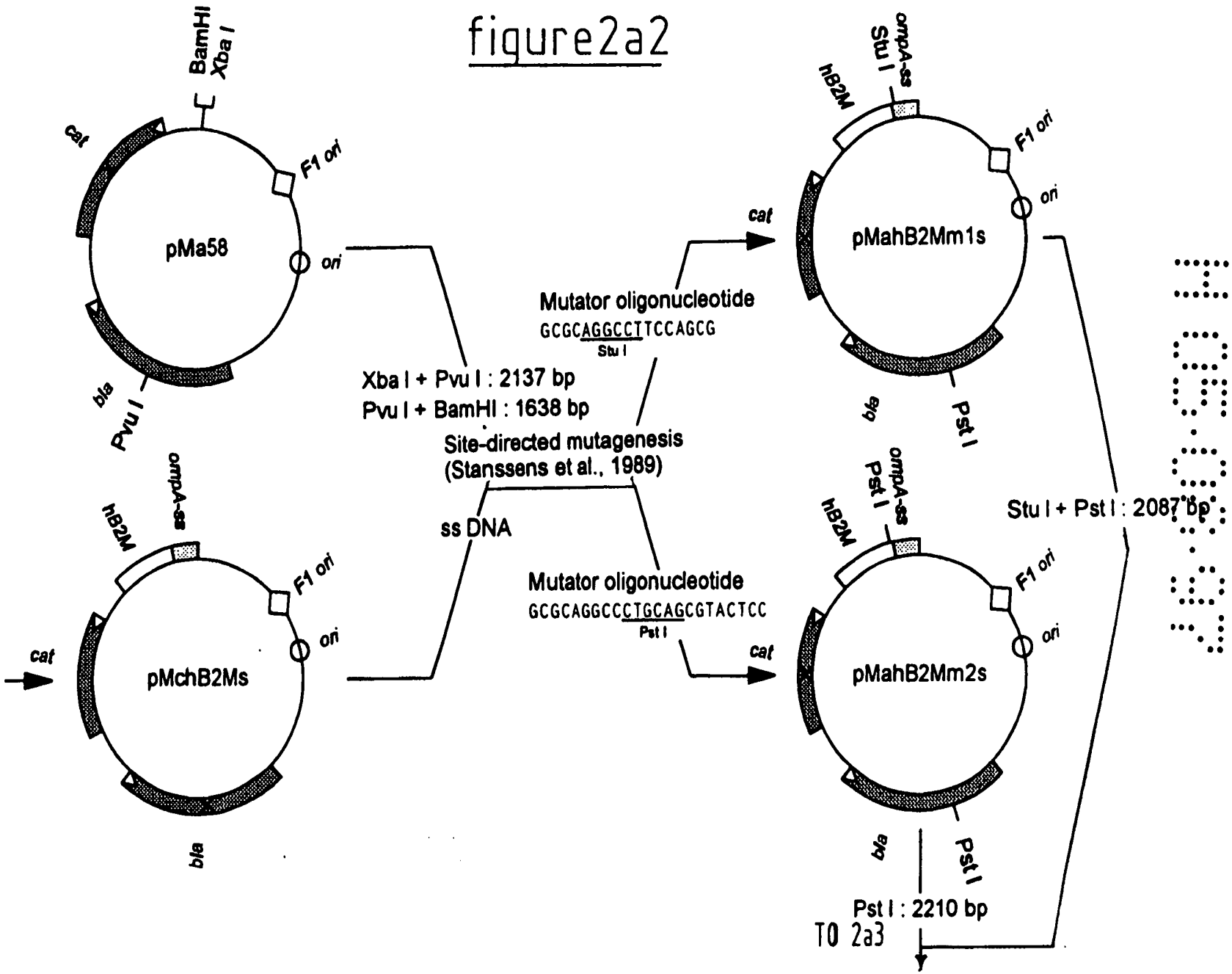
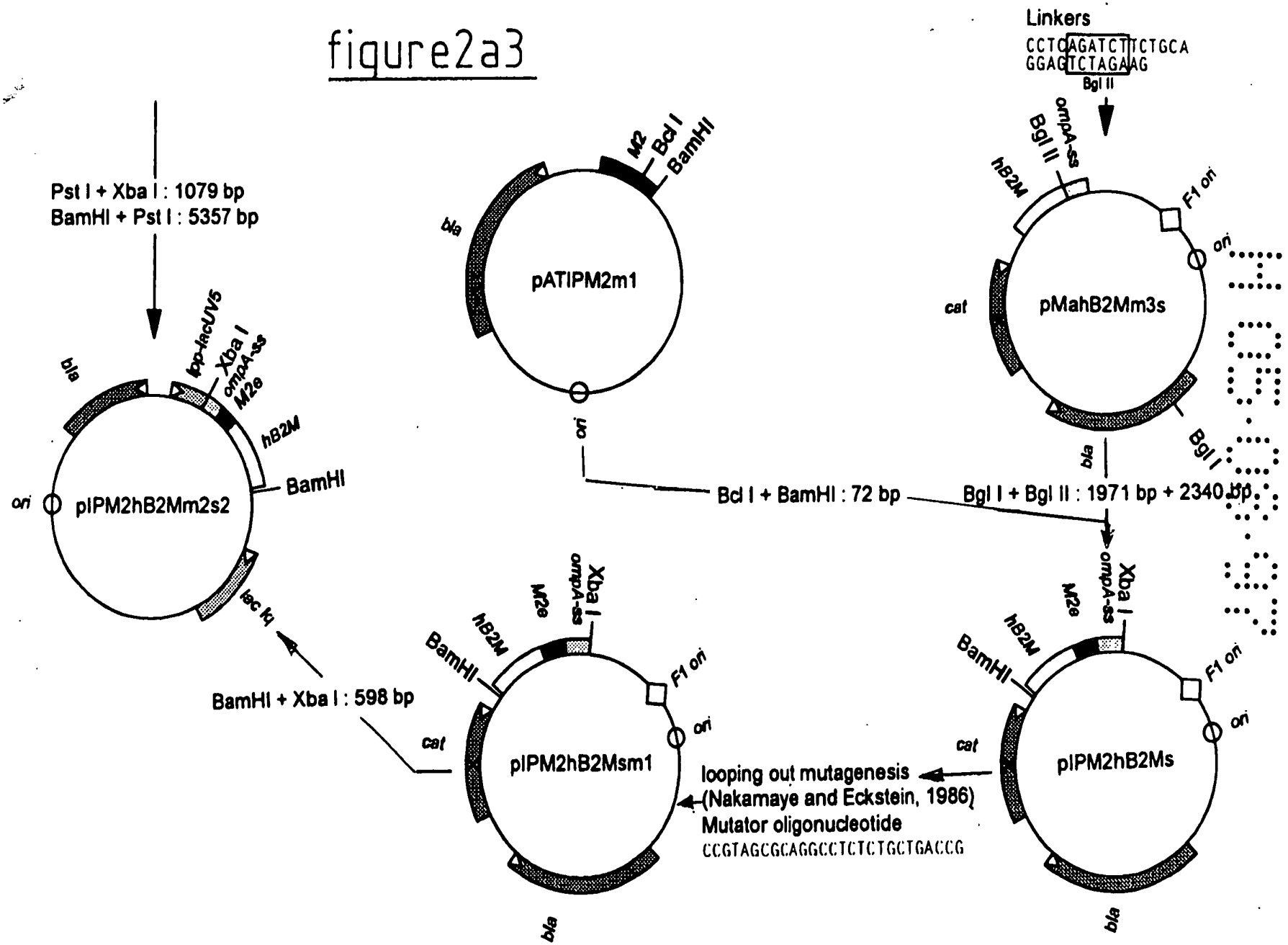


figure2a2



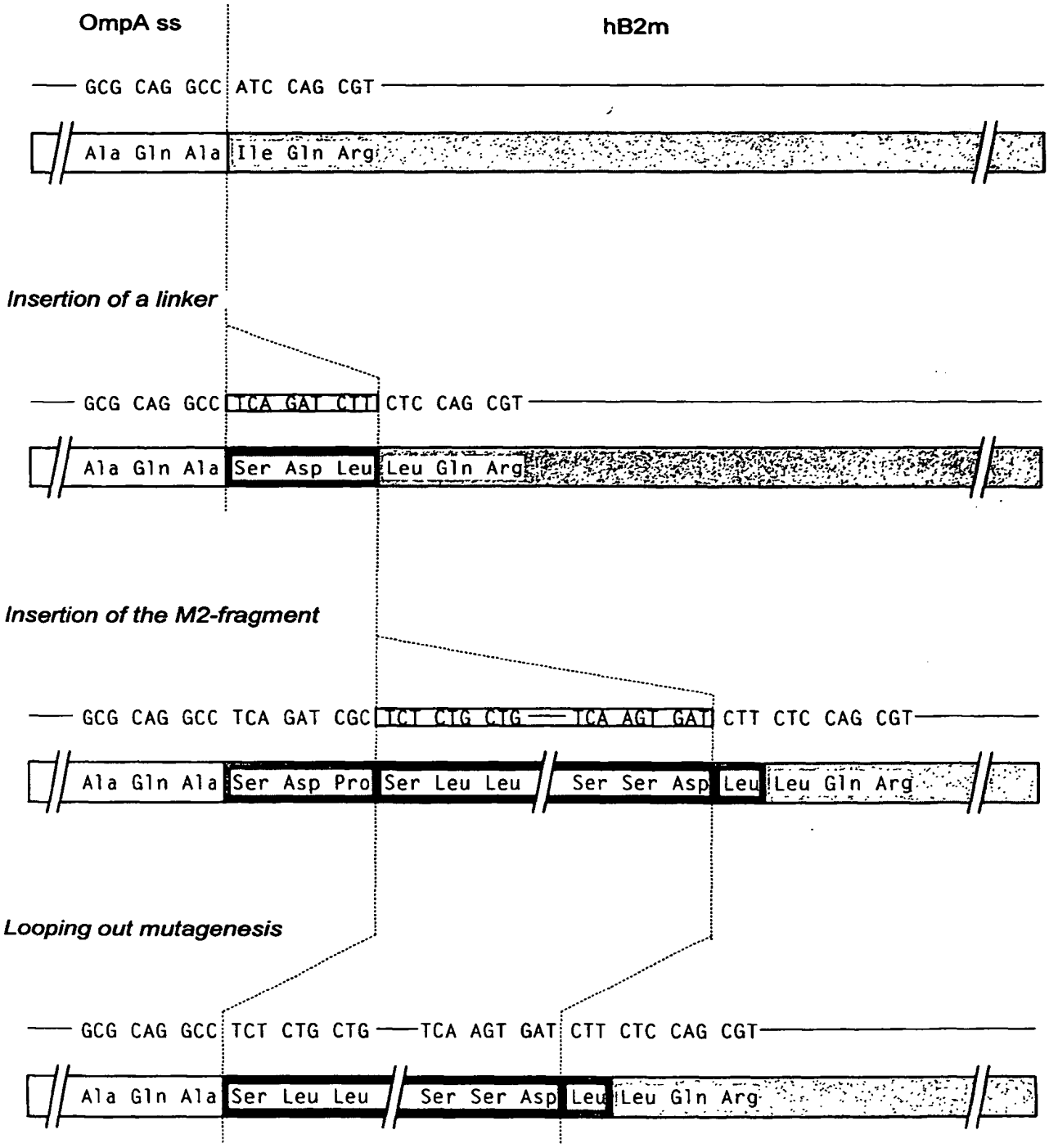
3/26

figure2a3


Linkers
CCTCAGATCTTCTGCA
GGAGTCTAGAAG
Bgl II
Pst I + Xba I : 1079 bp
BamHI + Pst I : 5357 bp
pATIPM2m1
M2
Bcl I
BamHI
bla
ori
pMahB2Mm3s
ompA-ss
Bgl II
hB2M
F1 ori
ori
cat
bla
Bgl I
pIPM2hB2Mm2s2
bla
tac-lacUV5
Xba I
ompA-ss
M2e
hB2M
BamHI
ori
lac Iq
Bcl I + BamHI : 72 bp
Bgl I + Bgl II : 1971 bp + 2340 bp
BamHI + Xba I : 598 bp
pIPM2hB2Msm1
pIPM2hB2Ms
looping out mutagenesis
(Nakamaye and Eckstein, 1986)
Mutator oligonucleotide
CCGTAGCGCAGGCCTCTCTGCTGACCG

H 05-08-97

Figure 2b



H 05.08.97

figure 3a1

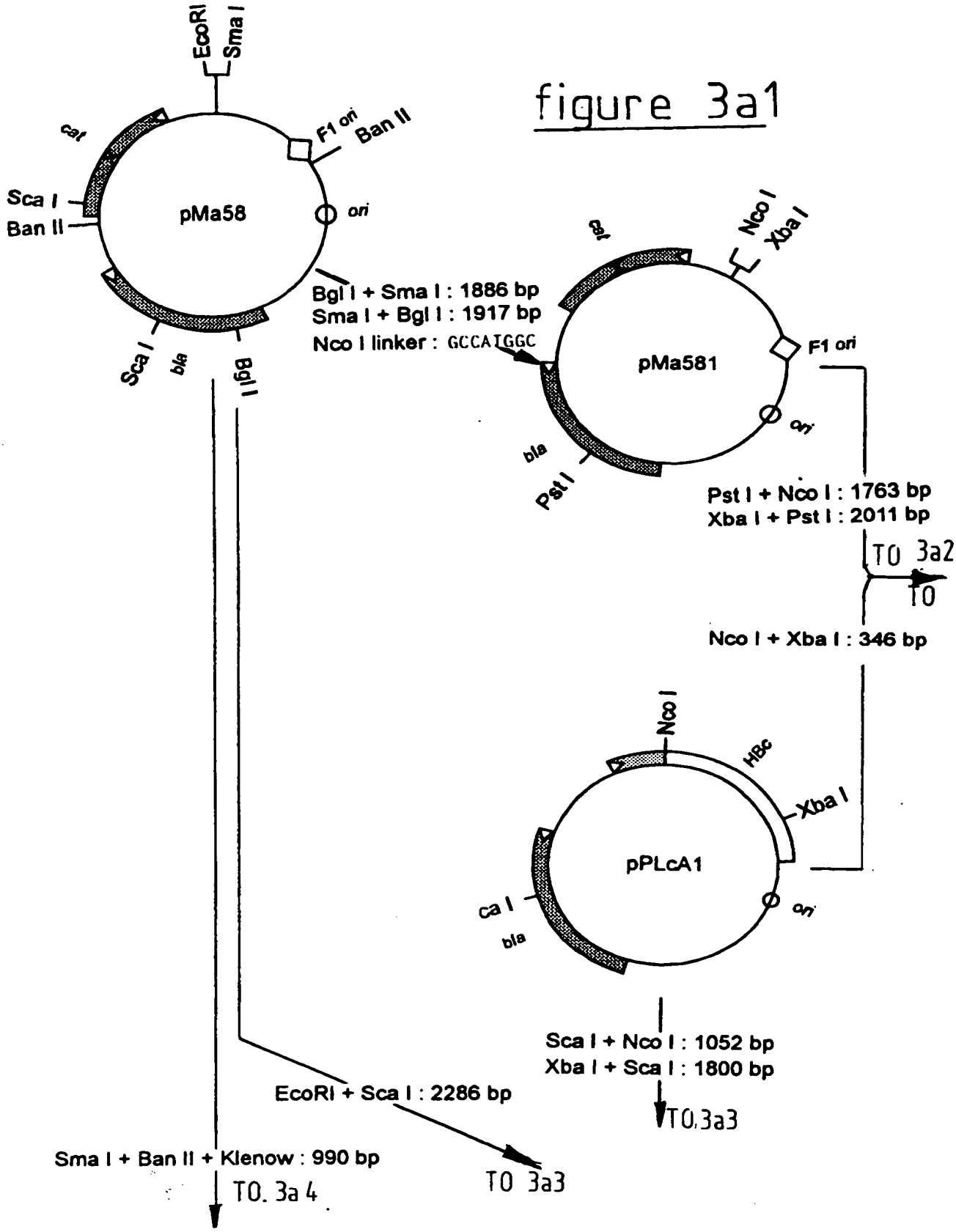


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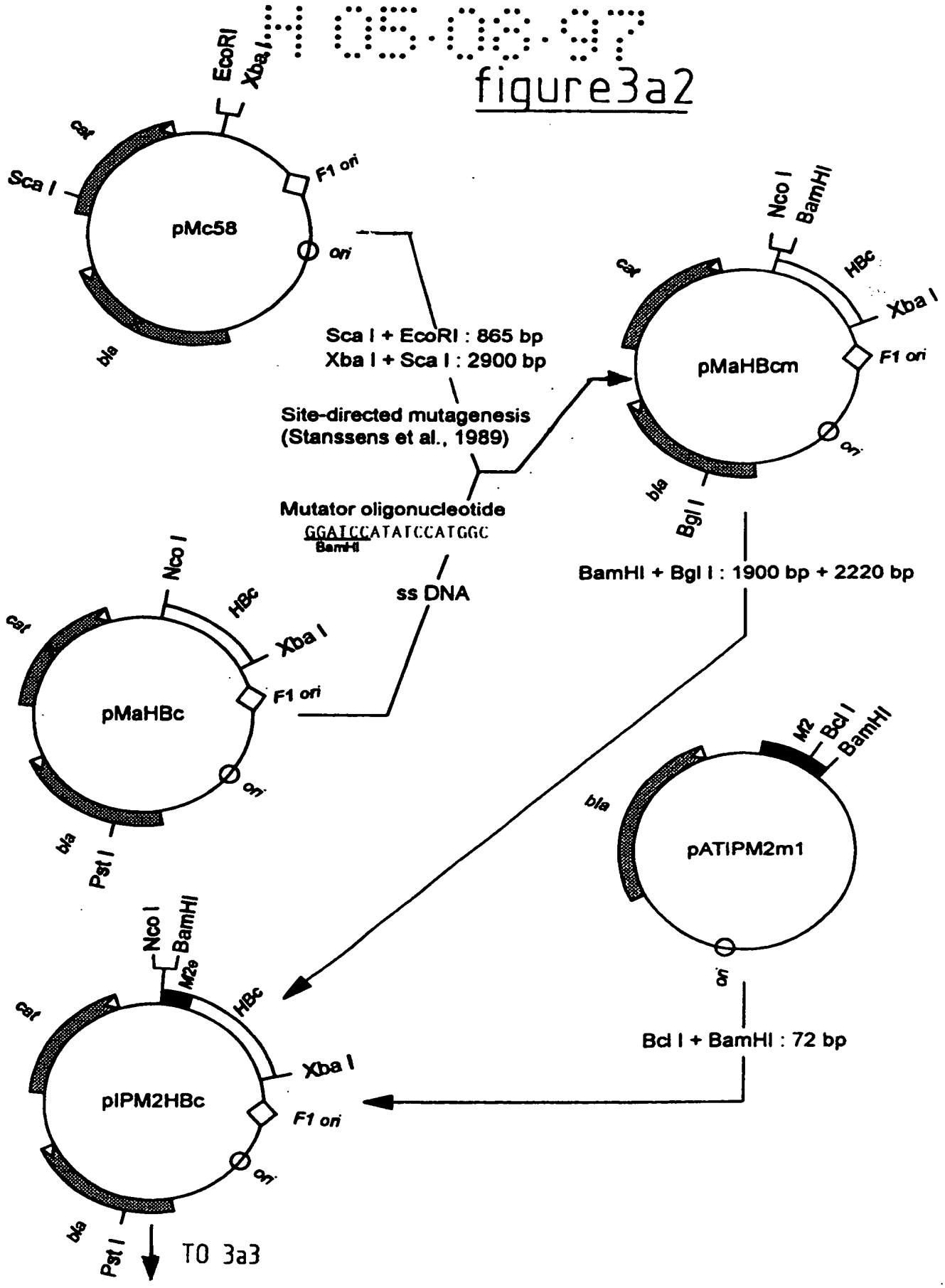
figure3a2



H 05-08-97

figure3a3

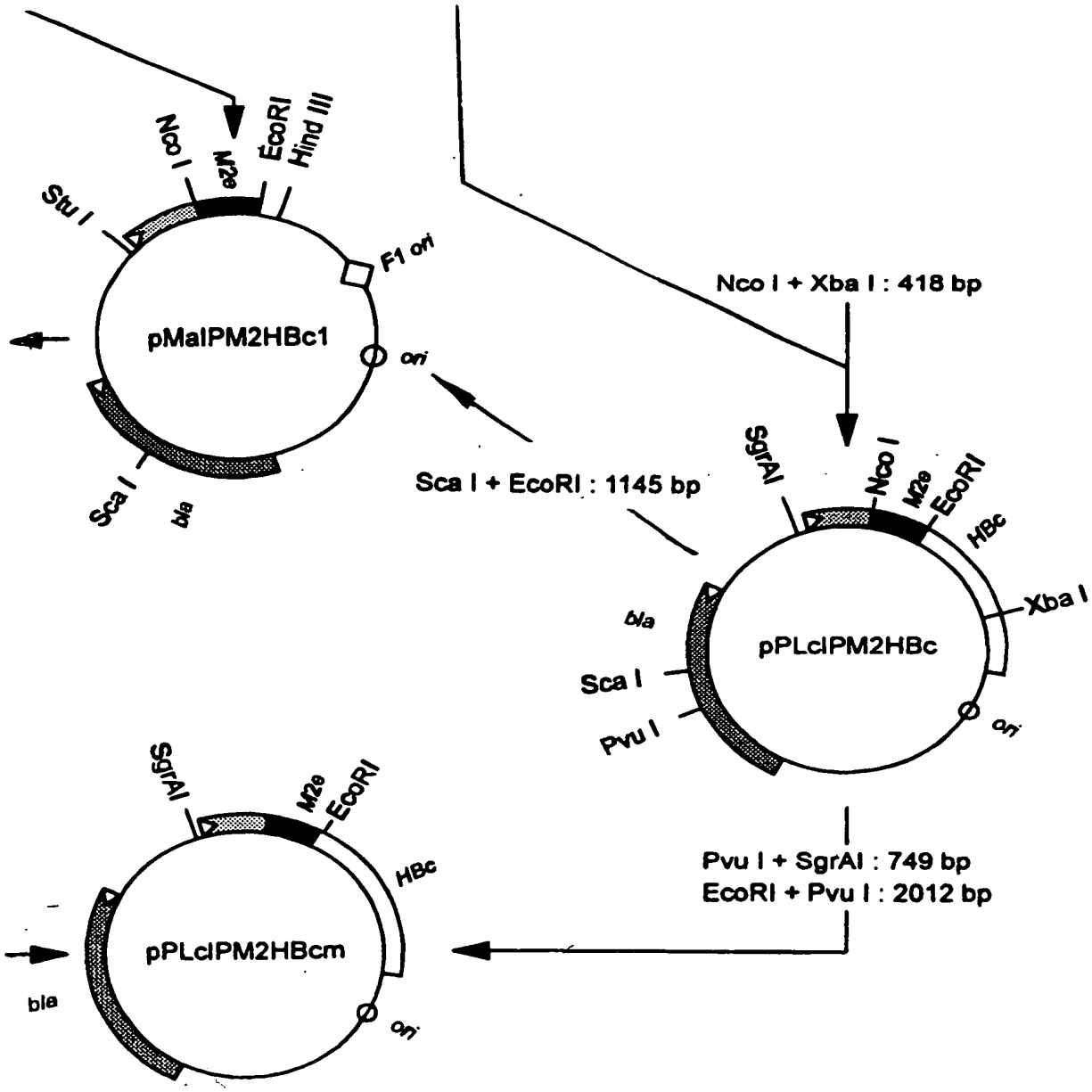


figure3a4

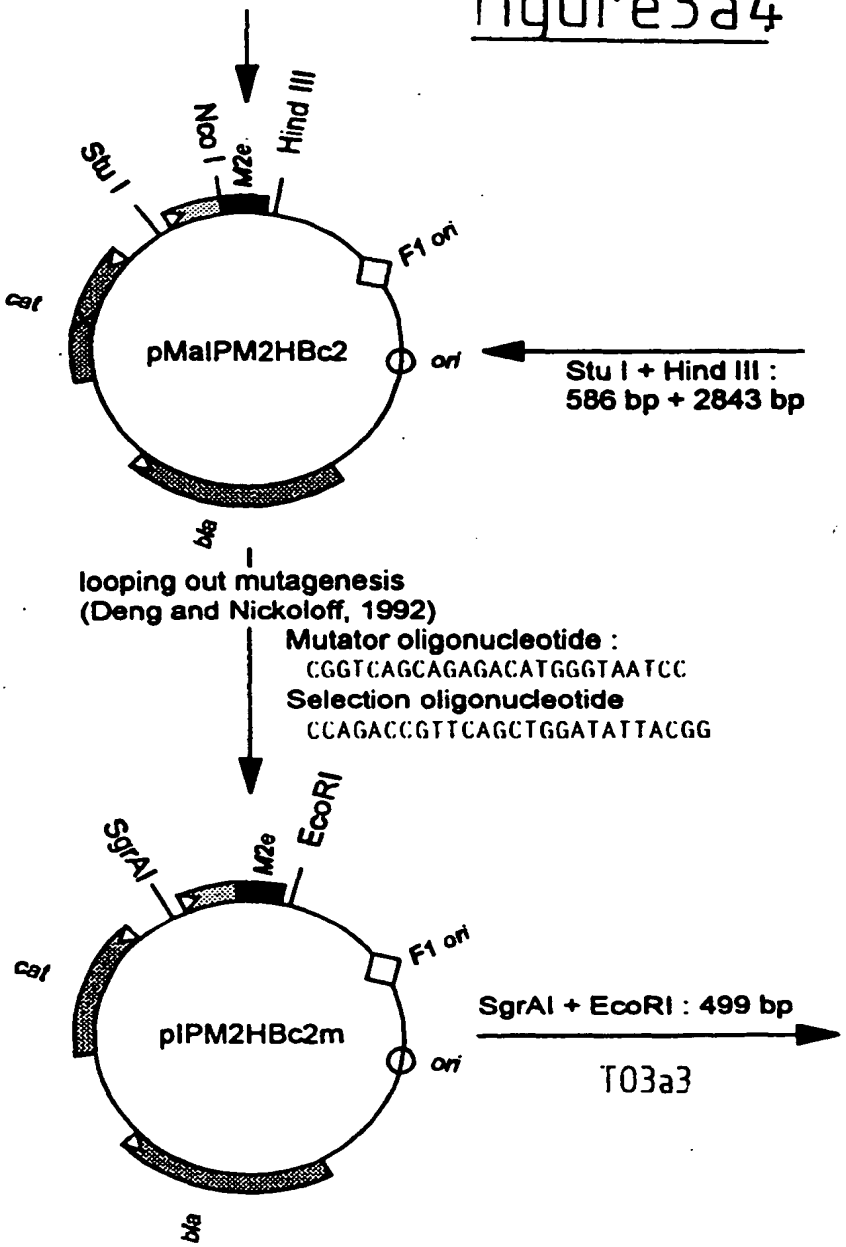


Figure 3b

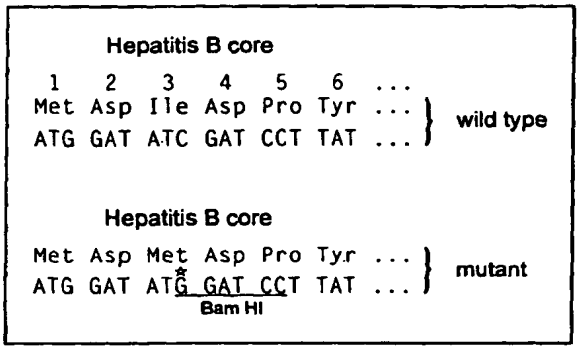
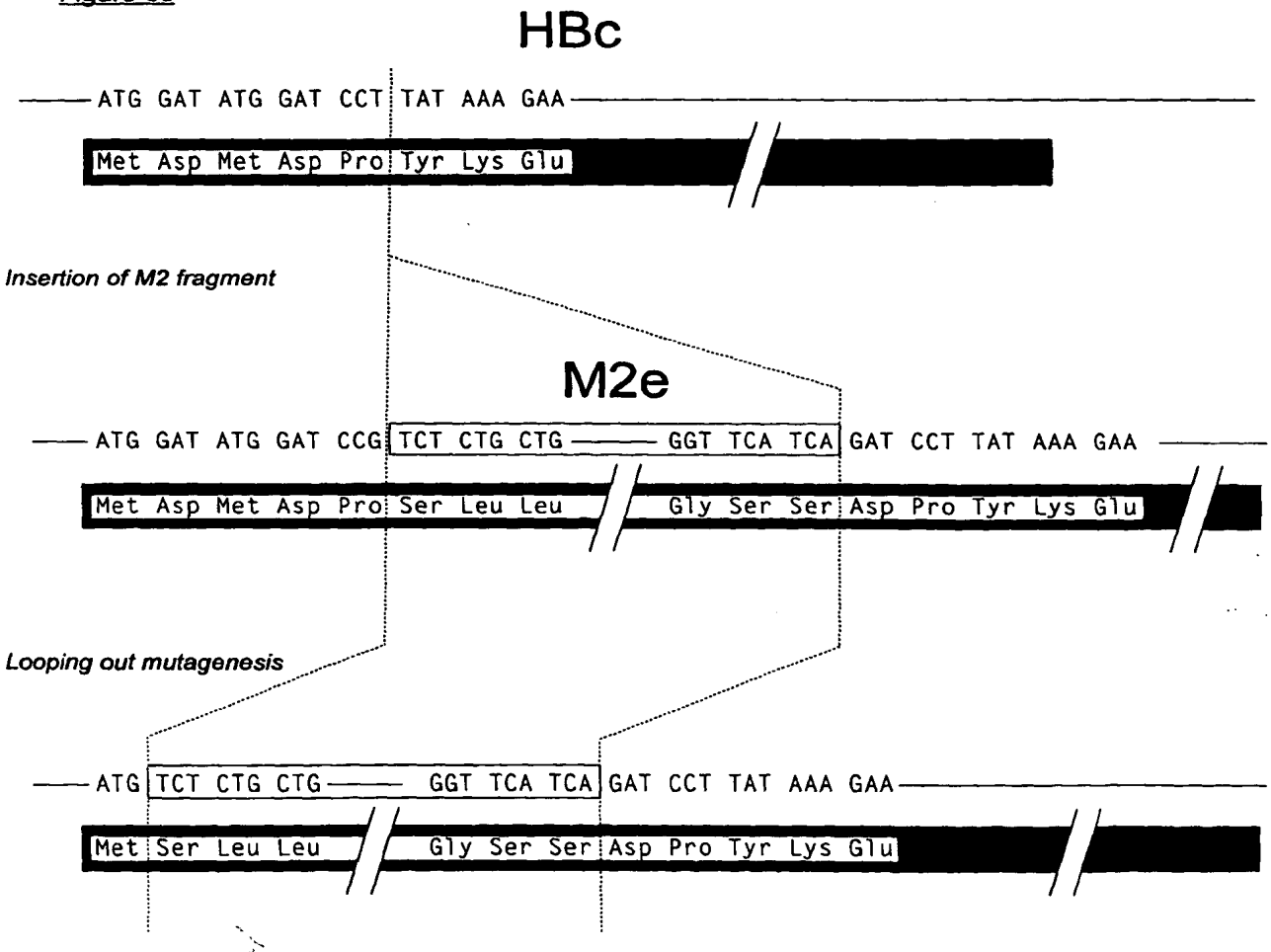


Figure 3c



11/26

Figure 4

H 05.08.97

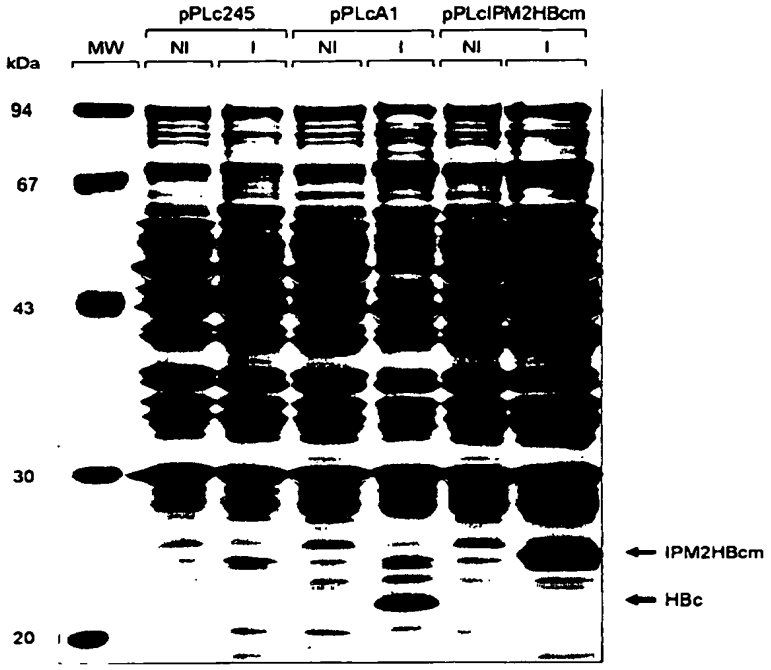


Figure 7

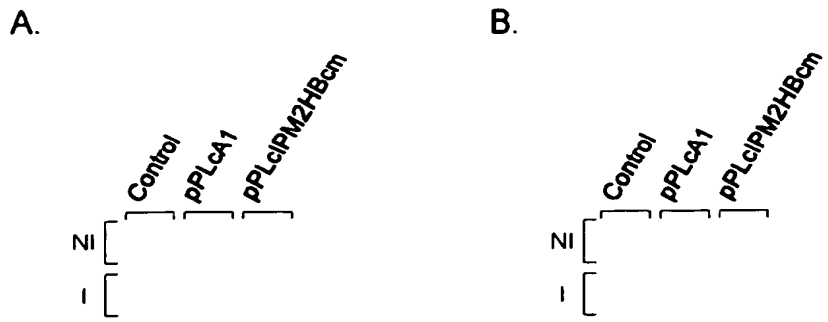
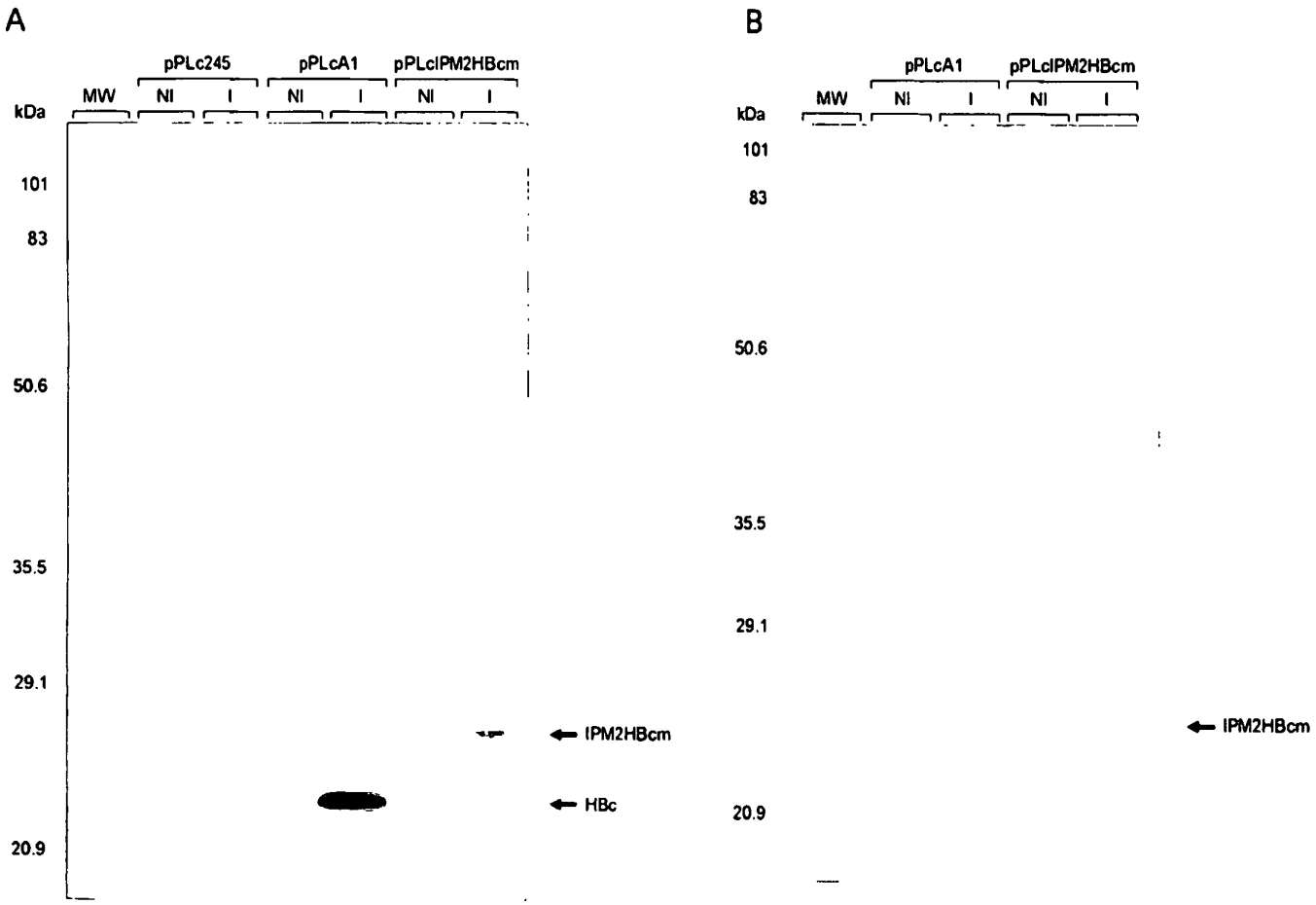


Figure 5



12/26

Figure 6

ATG TCT CTG CTG ACC GAA GTT GAA	Nucleotide sequence of *ipm2hbcm*
Met Ser Leu Leu Thr Glu Val Glu	Translated amino acid sequence
Ser Leu Leu Thr Glu Val Glu	Amino terminus of the fusion protein IPM2HBcm
Ser Leu Leu Thr Glu Val Glu	Amino terminus of the M2 protein of A/Udorn/72

Figure 8

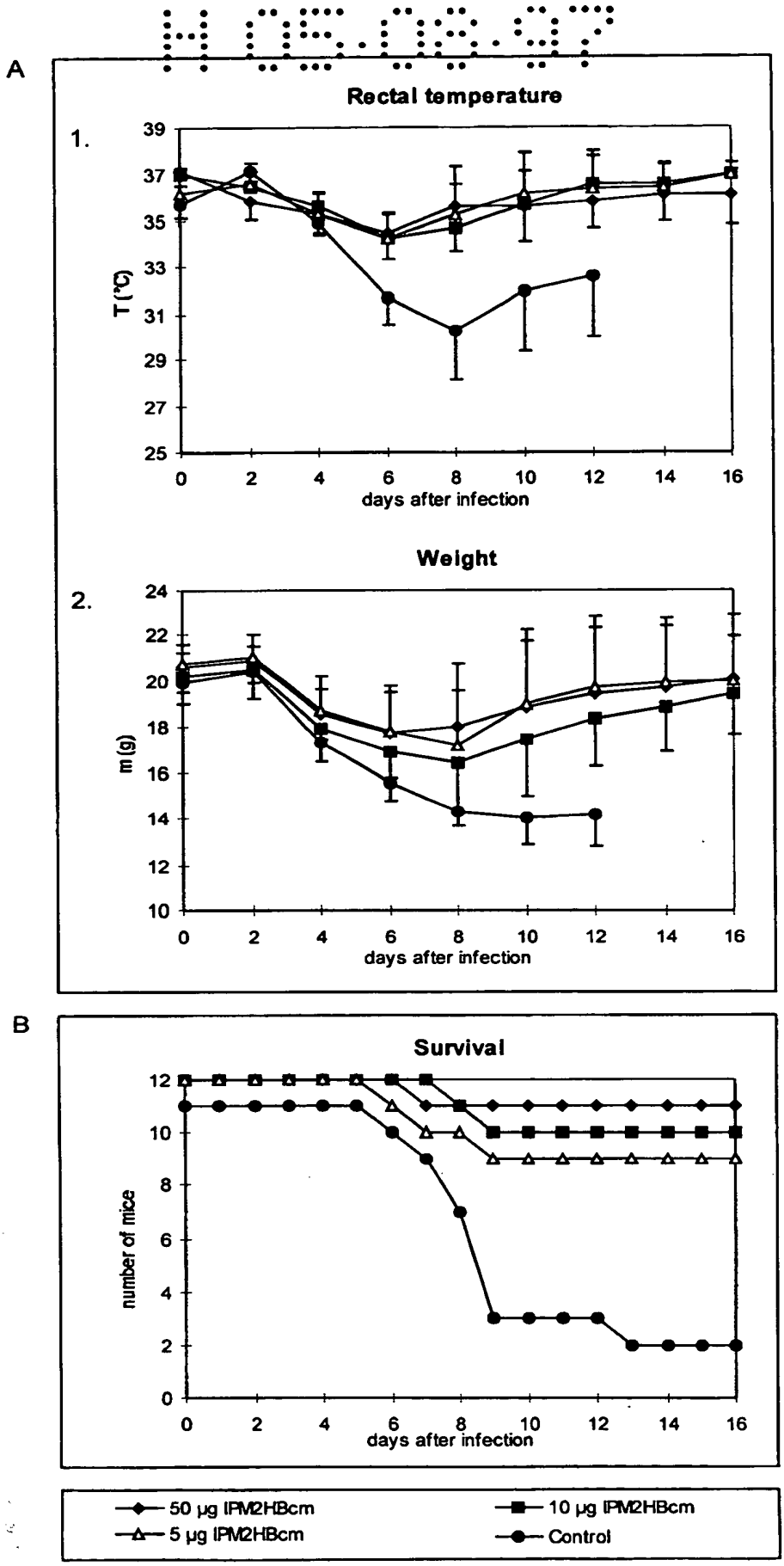

A
Rectal temperature
1.
T (°C)
39
37
35
33
31
29
27
25
0
2
4
6
8
10
12
14
16
days after infection
Weight
2.
m (g)
24
22
20
18
16
14
12
10
0
2
4
6
8
10
12
14
16
days after infection
B
Survival
number of mice
12
10
8
6
4
2
0
0
2
4
6
8
10
12
14
16
days after infection
50 µg IPM2HBcm
10 µg IPM2HBcm
5 µg IPM2HBcm
Control

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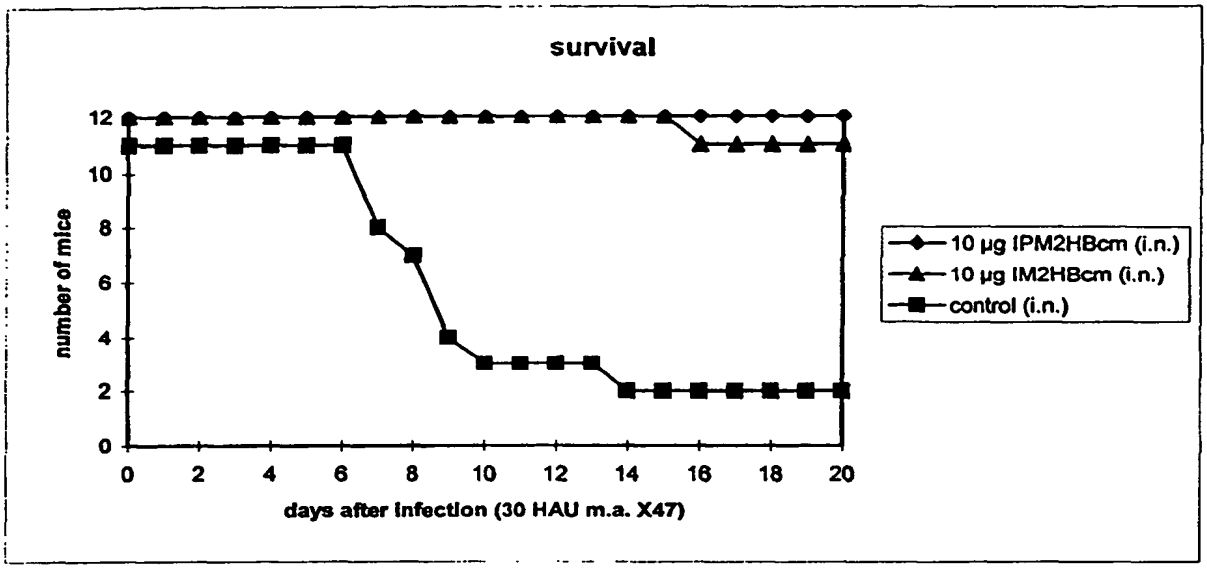
fig.8D



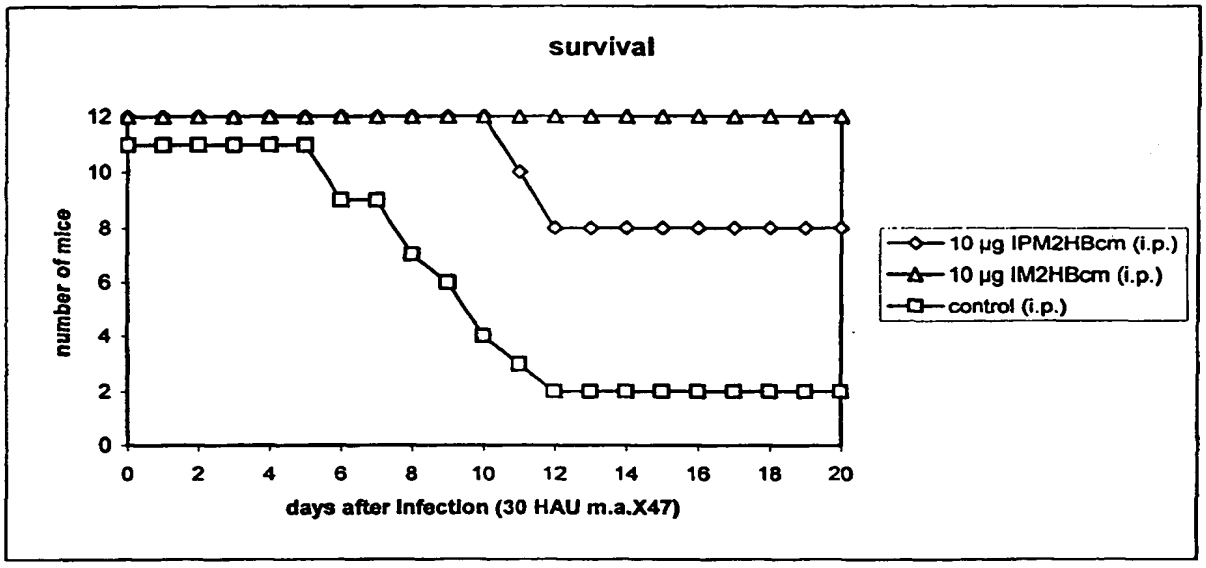


fig.8C

	surviving mice
10 µg IPM2HBcm (i.n.)	12/12
10 µg IM2HBcm (i.n.)	11/12
control (i.n.)	2/11
10 µg IPM2HBcm (i.p.)	8/12
10 µg IM2HBcm (i.p.)	12/12
control (i.p.)	2/12

Figure 9

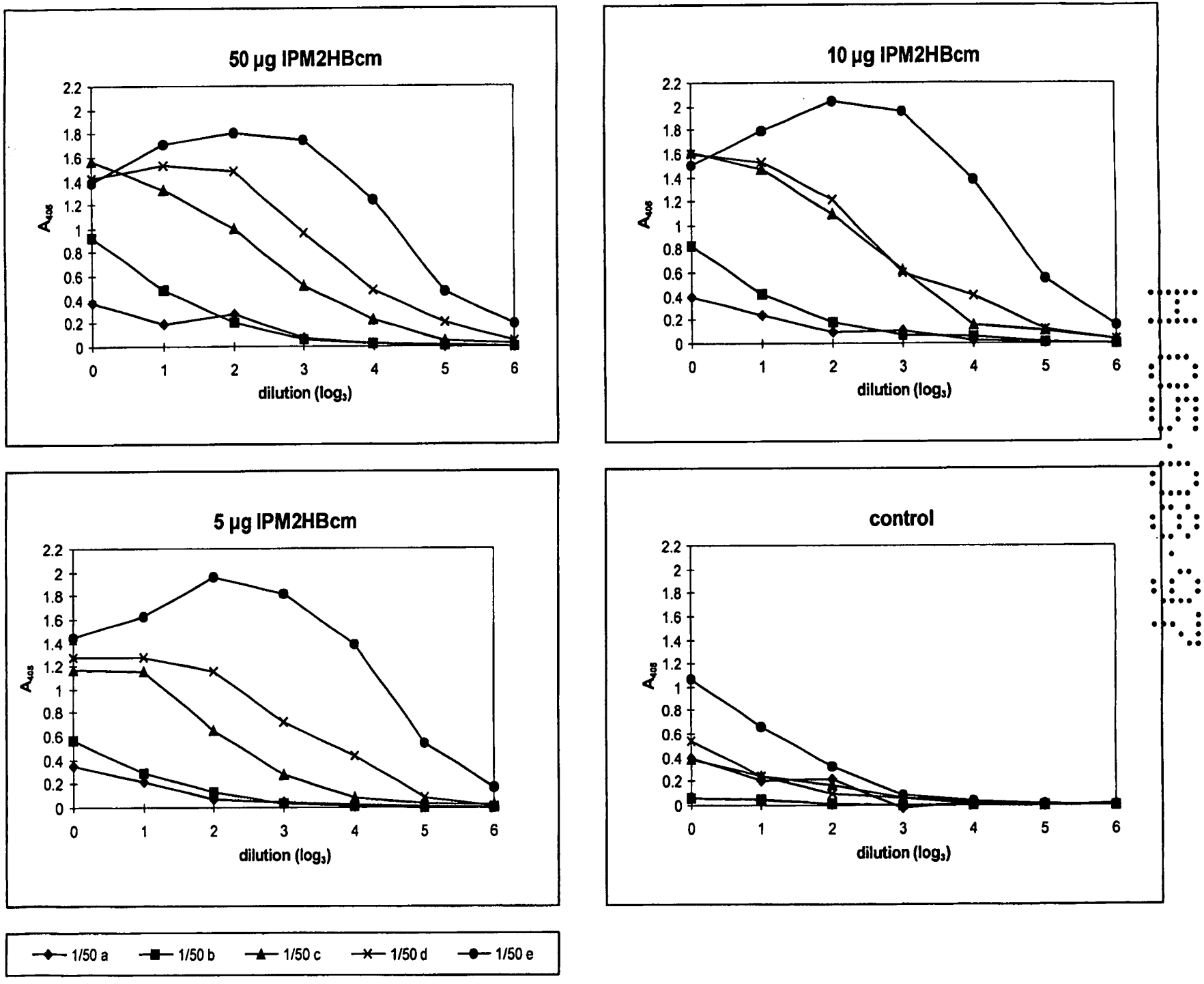
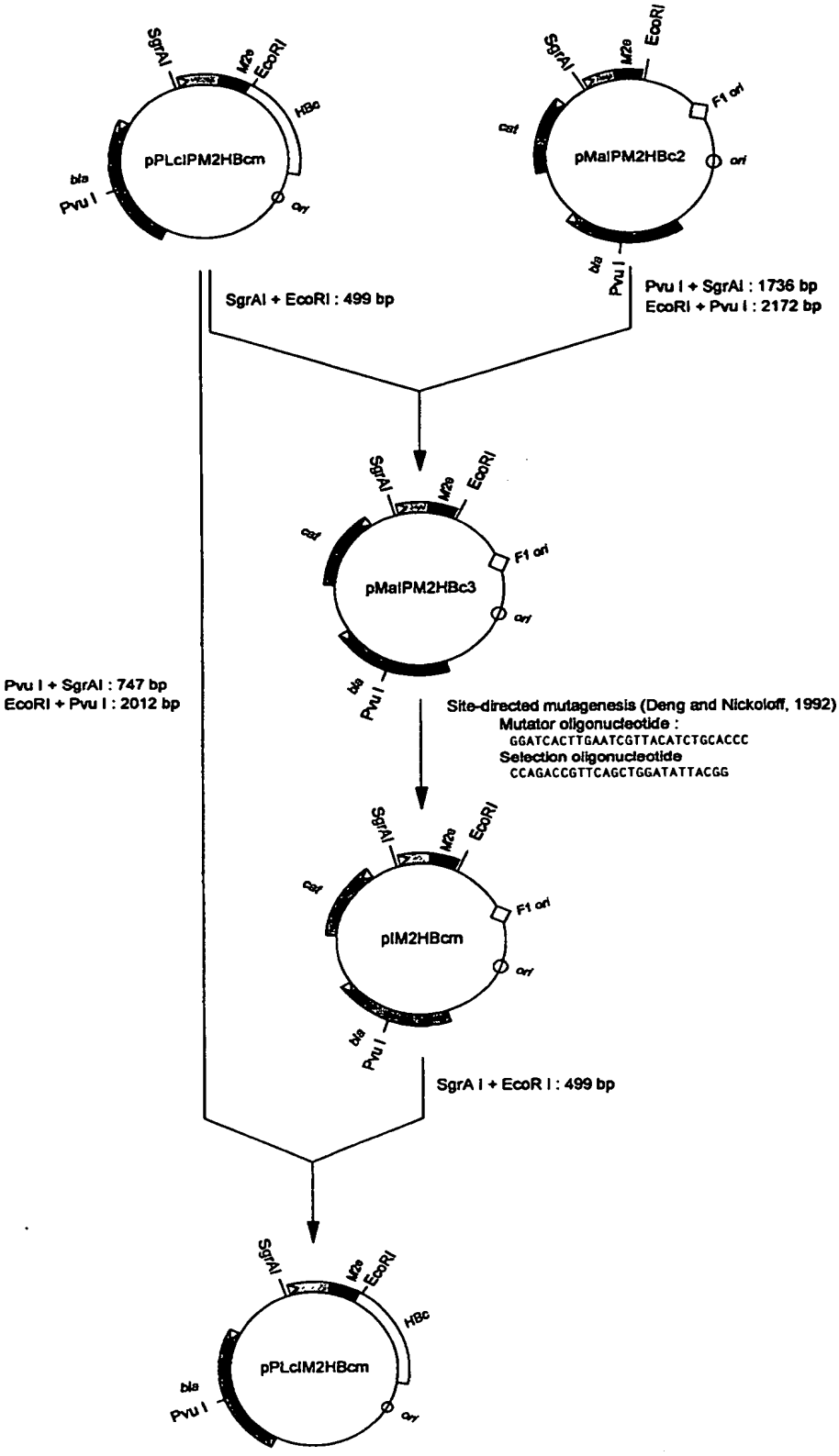

50 µg IPM2HBcm
10 µg IPM2HBcm
5 µg IPM2HBcm
control
A405
dilution (log3)
1/50 a
1/50 b
1/50 c
1/50 d
1/50 e

Figure 10



18/26

H 05-08-97

Figure 11

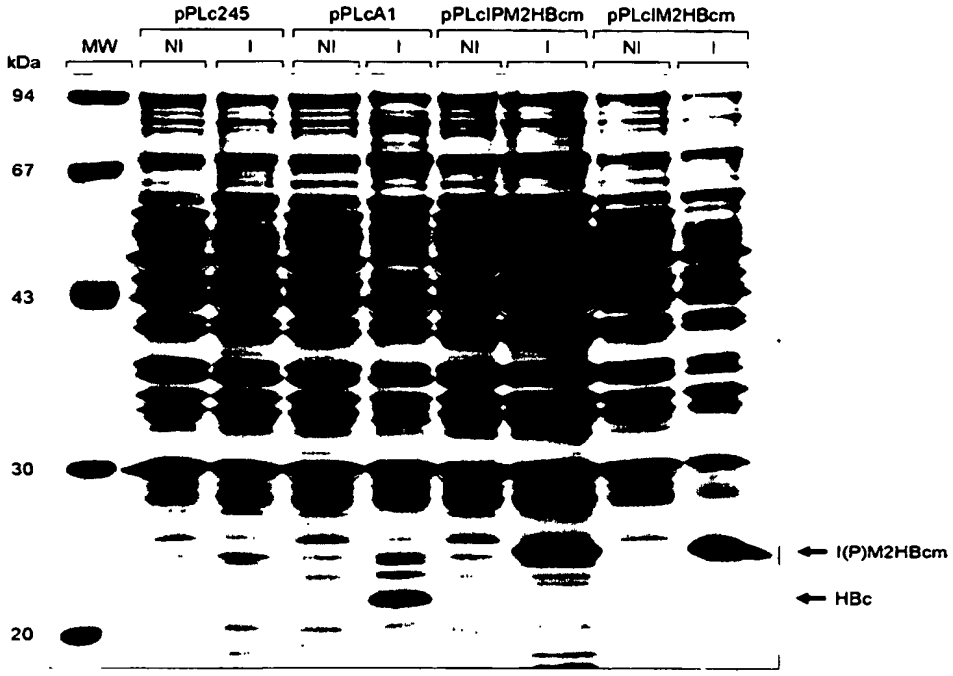


Figure 21

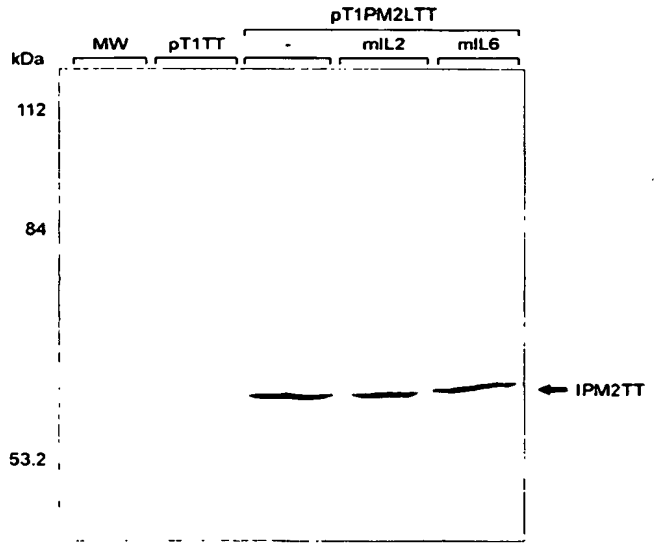
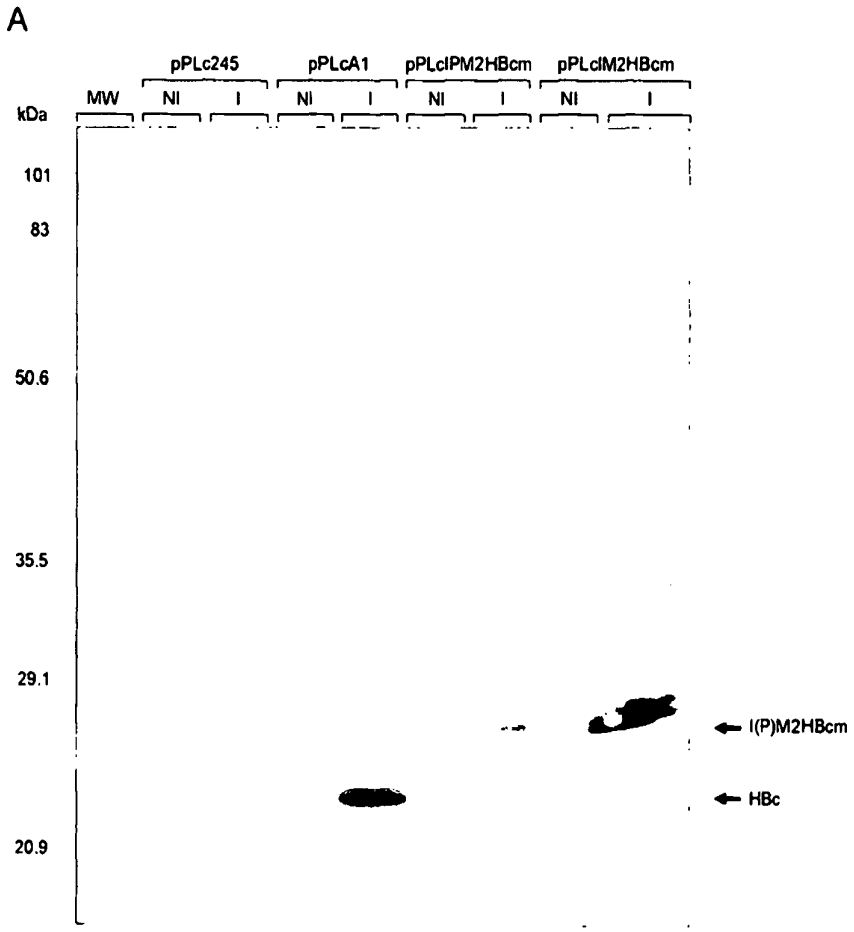


Figure 12


A
pPLc245
pPLcA1
pPLclPM2HBcm
pPLclM2HBcm
MW
NI
I
kDa
101
83
50.6
35.5
29.1
20.9
I(P)M2HBcm
HBc

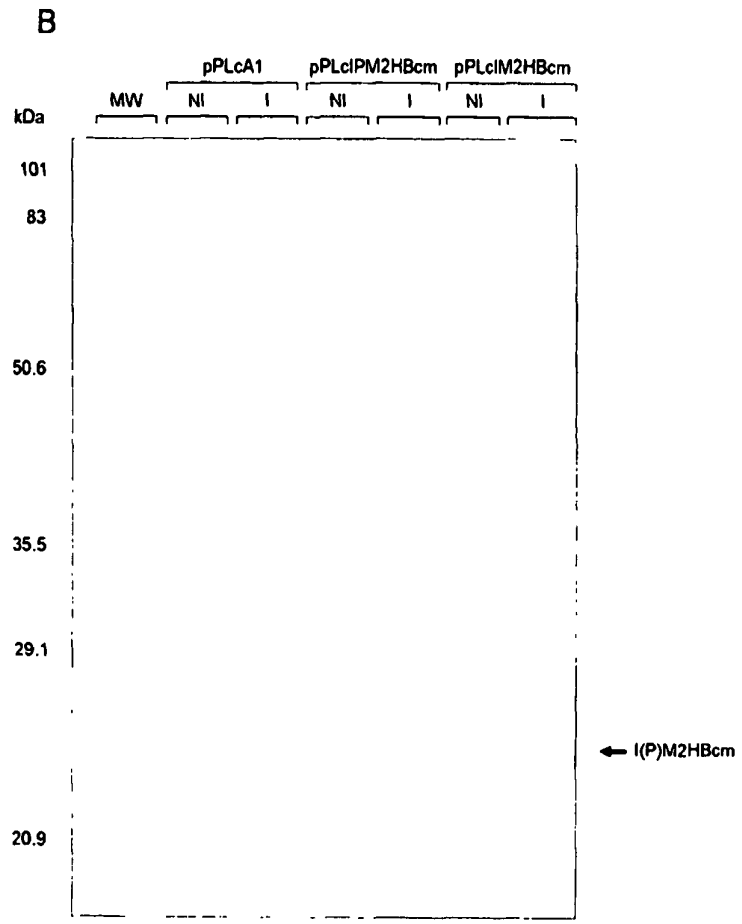

B
pPLcA1
pPLclPM2HBcm
pPLclM2HBcm
MW
NI
I
kDa
101
83
50.6
35.5
29.1
20.9
I(P)M2HBcm

Figure 13

HBcs (27-mer)

CATGGATATGGATCCTTATAAAGAATT
start

M2s (23-mer)

CATGTCTCTGCTGACCGAAGTTG
start

M2Ls (29-mer)

CATGTCTTTATTAACCGAAGTTGAAACCC
start

HBca (39-mer)

CGTGATCAACTAGTTCACTAACATTGAGATTCCCGAGAT
BclI SpeI stop

Figure 14

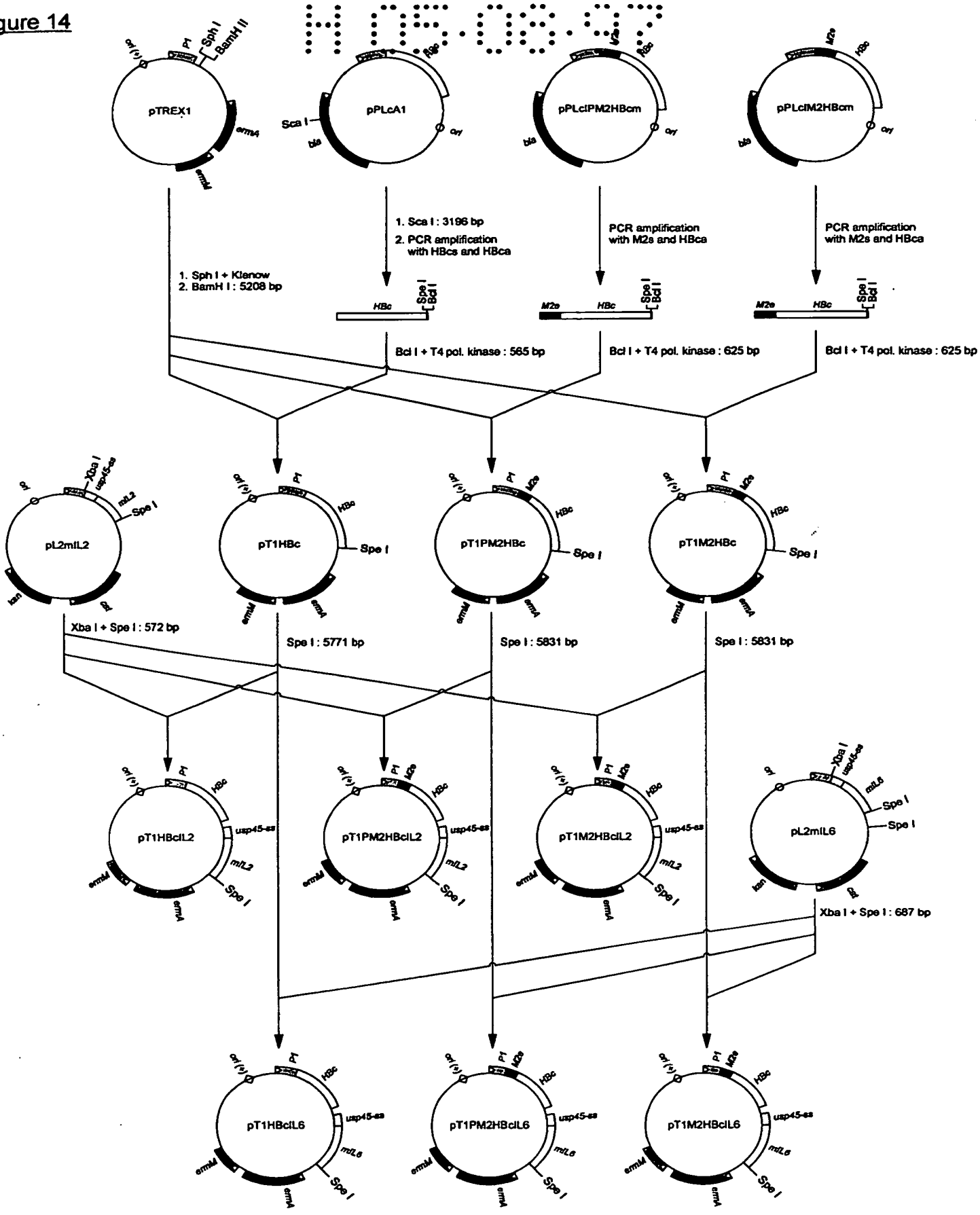

pTREX1
pPLcA1
pPLclPM2HBcm
pPLclM2HBcm
1. Sca I : 3196 bp
2. PCR amplification with HBcs and HBca
PCR amplification with M2s and HBca
PCR amplification with M2s and HBca
1. Sph I + Klenow
2. BamH I : 5208 bp
Bcl I + T4 pol. kinase : 565 bp
Bcl I + T4 pol. kinase : 625 bp
Bcl I + T4 pol. kinase : 625 bp
pL2mIL2
pT1HBc
pT1PM2HBc
pT1M2HBc
Xba I + Spe I : 572 bp
Spe I : 5771 bp
Spe I : 5831 bp
Spe I : 5831 bp
pT1HBcIL2
pT1PM2HBcIL2
pT1M2HBcIL2
pL2mIL6
Xba I + Spe I : 687 bp
pT1HBcIL6
pT1PM2HBcIL6
pT1M2HBcIL6

Figure 15

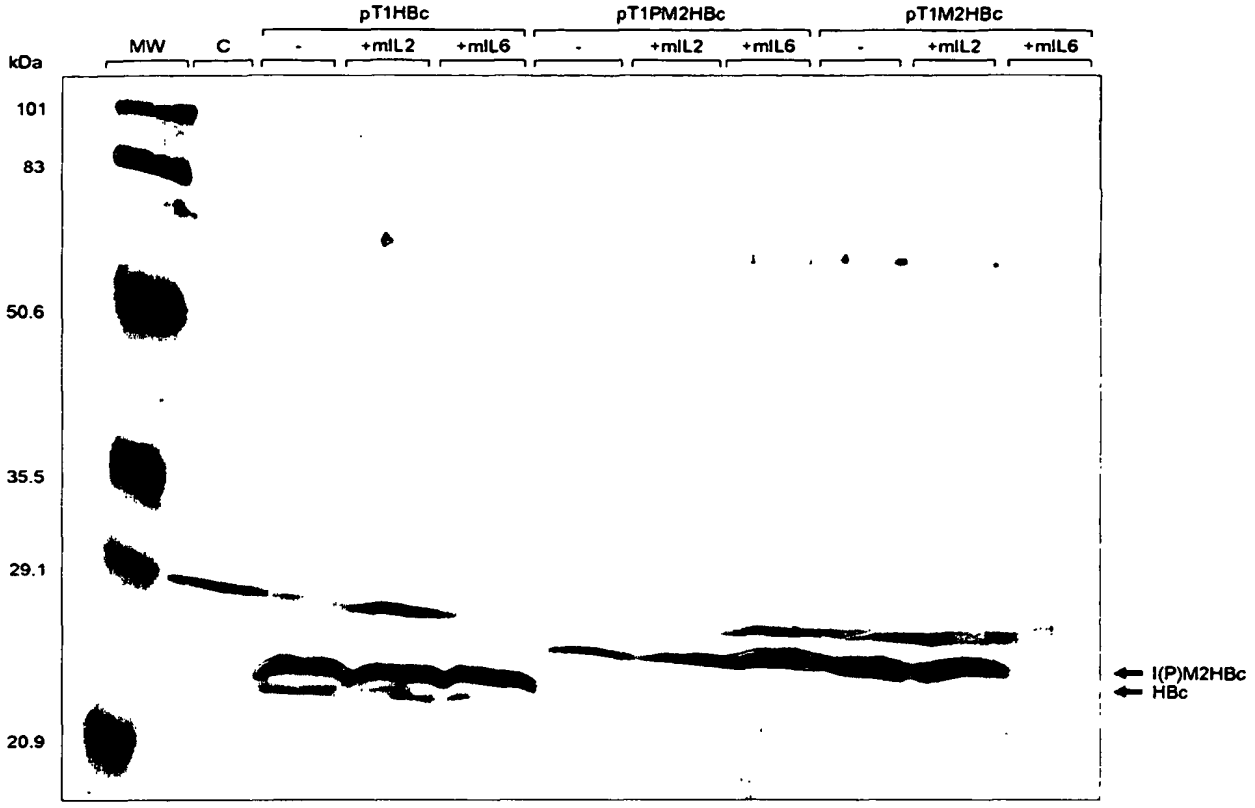


Figure 16

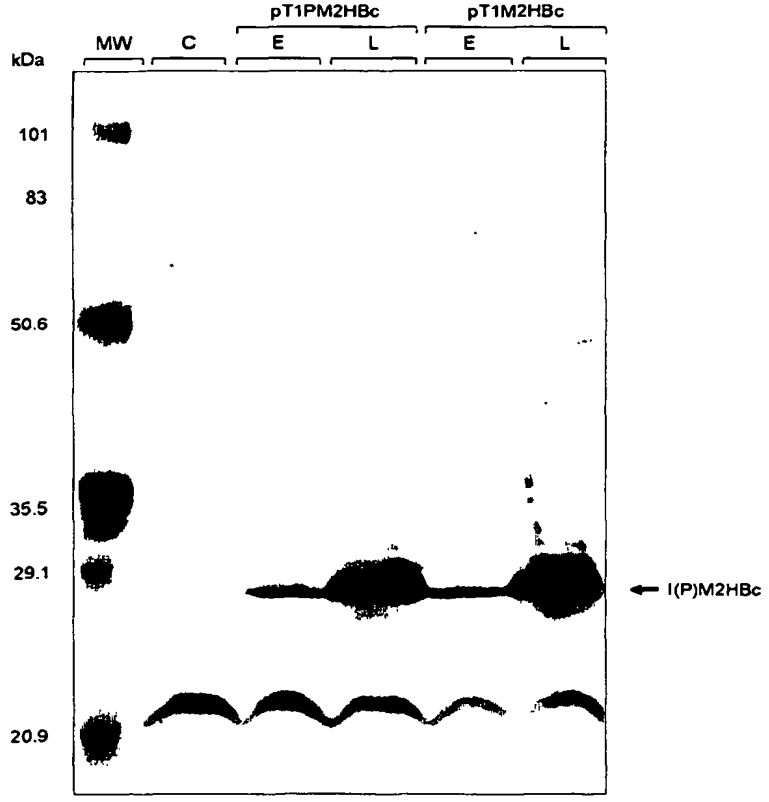


Figure 17

M2Ca (33-mer)

CGGGATCCCCACTTGAATCGTTACATCTGCACC
BamH I

M2LSs (30-mer)

TCTTTATTAACCGAAGTTGAAACCCCTATC
Ser

C3ds (35-mer)

CCGCGCCCACCCGACGAGATCTCGGATCTACCCCC
Bgl II

C3da (38-mer)

GCACTAGTTCAAGGATCCGATCCGAACTCTTCAGATCC
Spe I stop BamH I

Figure 18

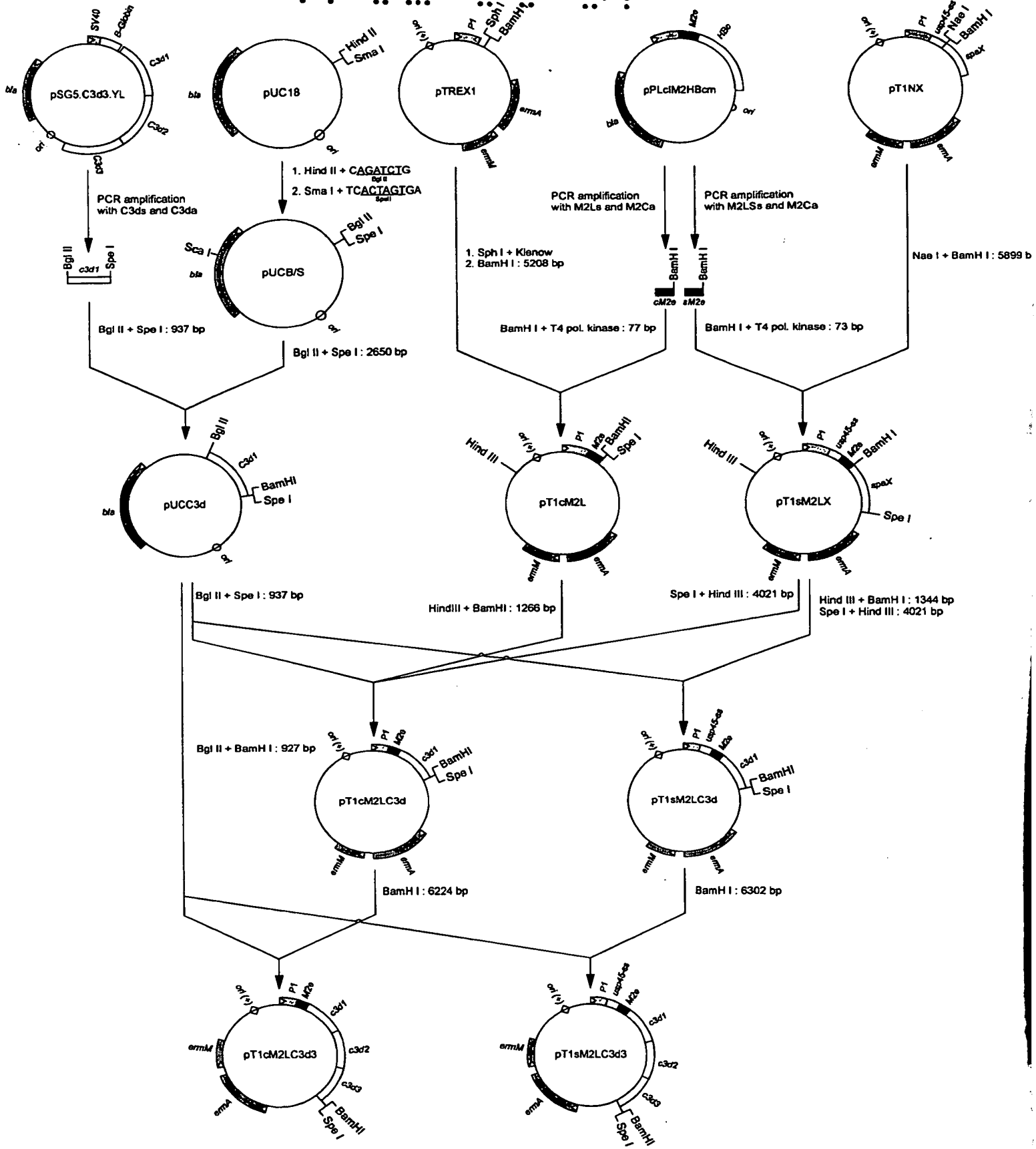

pSG5.C3d3.YL
SV40
β-Globin
C3d1
C3d2
C3d3
bla
ori
pUC18
Hind II
Sma I
pTREX1
ori (+)
P1
Sph I
BamH I
ermA
ermM
pPLclM2HBcm
M2e
HBe
cry
pT1NX
Nae I
spaX
1. Hind II + CAGATCTG
2. Sma I + TCACTAGTGA
PCR amplification with C3ds and C3da
PCR amplification with M2Ls and M2Ca
PCR amplification with M2LSs and M2Ca
Bgl II
Spe I
c3d1
Sca I
pUCB/S
1. Sph I + Klenow
2. BamH I : 5208 bp
cM2e
sM2e
Nae I + BamH I : 5899 b
Bgl II + Spe I : 937 bp
Bgl II + Spe I : 2650 bp
BamH I + T4 pol. kinase : 77 bp
BamH I + T4 pol. kinase : 73 bp
pUCC3d
Hind III
pT1cM2L
pT1sM2LX
Spe I + Hind III : 4021 bp
Hind III + BamH I : 1344 bp
Spe I + Hind III : 4021 bp
HindIII + BamHI : 1266 bp
Bgl II + BamH I : 927 bp
pT1cM2LC3d
pT1sM2LC3d
BamH I : 6224 bp
BamH I : 6302 bp
pT1cM2LC3d3
pT1sM2LC3d3

Figure 19

H 05.08.97

TTFCs (35-mer)

CGGGATCCGACACCAATTCCATTTTCTTATTCTAA
BamH I

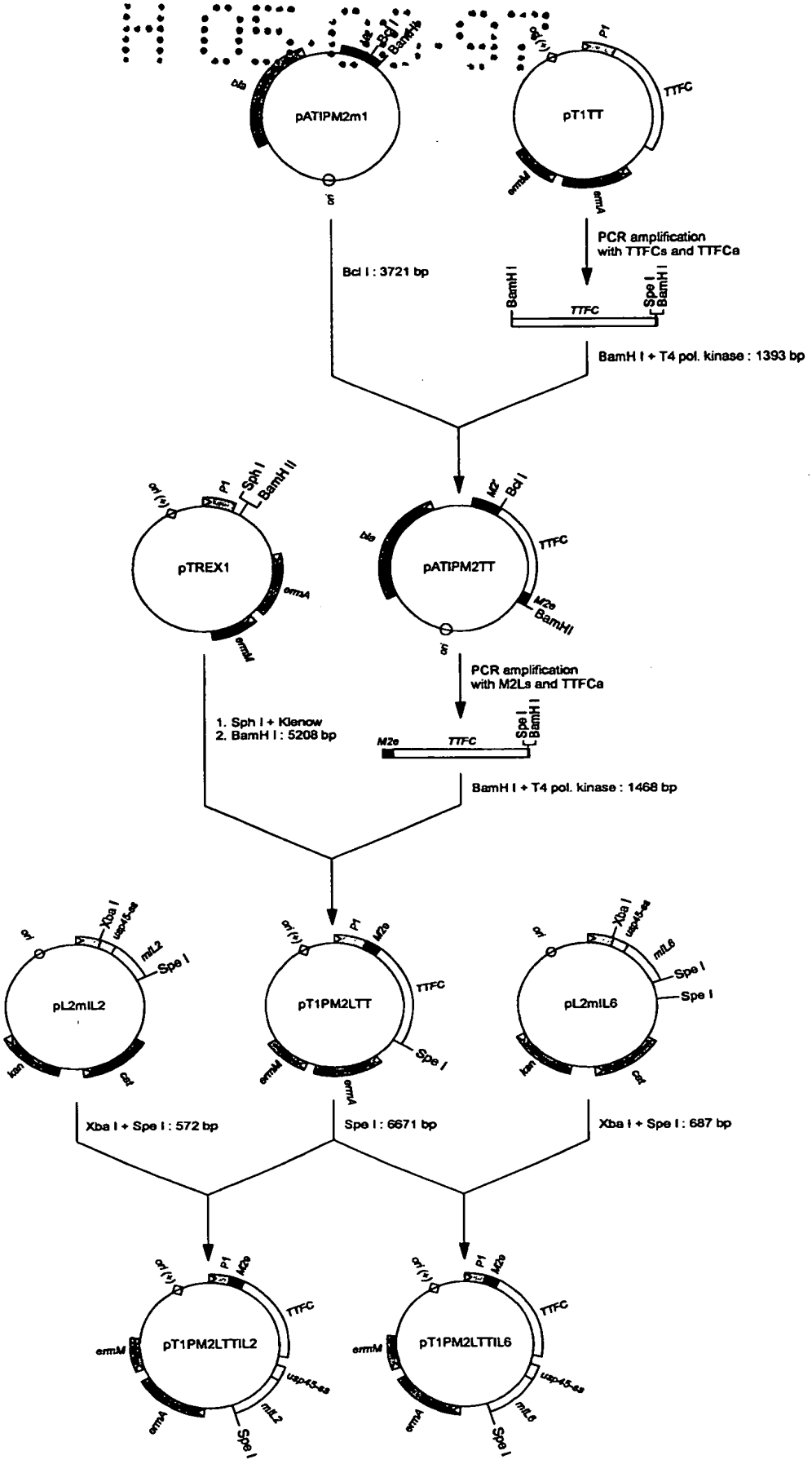
TTFCa (25-mer)

GGGGATCCACTAGTTTAATCATTTG
Bcl I Spe I stop

M2Ls (29-mer)

CATGTCTTTATTAACCGAAGTTGAAACCC
start

Figure 20


pATIPM2m1
pT1TT
PCR amplification
with TTFCs and TTFCa
Bcl I : 3721 bp
BamH I + T4 pol. kinase : 1393 bp
pTREX1
pATIPM2TT
PCR amplification
with M2Ls and TTFCa
1. Sph I + Klenow
2. BamH I : 5208 bp
BamH I + T4 pol. kinase : 1468 bp
pL2mIL2
pT1PM2LTT
pL2mIL6
Xba I + Spe I : 572 bp
Spe I : 6671 bp
Xba I + Spe I : 687 bp
pT1PM2LTTIL2
pT1PM2LTTIL6

ABSTRACT

The present invention relates to an influenza antigen, comprising a fusion product of at least the extracellular part of a conserved influenza membrane protein or a functional fragment thereof and a presenting carrier, which may be a presenting (poly)peptide or a non-peptidic structure, such as glycans, peptide mimetics, synthetic polymers. The invention further relates to a vaccine against influenza, comprising at least an antigen of the invention, optionally in the presence of one or more excipients. The invention also relates to use of the antigen, a method for preparing the antigen and acceptor cells expressing the antigen.